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Effective Drug Treatment Induces Drug Resistance Through Rapid Genome Alteration-Mediated Cancer Evolution

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EFFECTIVE DRUG TREATMENT INDUCES DRUG RESISTANCE THROUGH RAPID GENOME ALTERATION-MEDIATED CANCER EVOLUTION

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For Candice, River and Miles

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CHAPTER 1: INTRODUCTION

Cancer drug resistance represents a significant challenge in current cancer therapeutics

Systemic chemotherapeutics for metastatic and hematologic cancer patients were introduced as early as the 1940s (DeVita Jr and Chu 2008). Over a hundred drugs have since been designed and approved to treat various cancers (Baldo and Pham 2013), with mechanisms ranging from disrupting general cellular processes to targeting cancer-specific molecular markers, and chemotherapy is currently one of the principal methods of cancer treatment. The key rationale behind the development of these various agents is to attack rapidly dividing tumor cells from multiple angles. We are currently in a "War on Cancer," and the generally accepted strategy to win this war and destroy the enemy is to aggressively eliminate as many tumor cells as possible with the most powerful forces possible (e.g. high-dose chemotherapeutics) (Heng 2015).

There have been advances in terms of extending patient lifespan and exceptional curative cases, ranging from the first effective combination chemotherapy programs for acute childhood leukemia, advanced Hodgkin's disease and metastatic testicular cancer (Li, Whitmore et al. 1960; DeVita Jr and Chu 2008), to the specific molecular targeting triumphs of chronic myeloid leukemia in the chronic phase and Pml-Rara-positive acute promyelocytic leukemia (Druker, Tamura et al. 1996; Druker, Sawyers et al. 2001; Hu, Liu et al. 2009). Unfortunately, with exception of a very low minority of "lucky" patients with greatly improved survival (many cancer types/regimens/agents have their

exceptional cases), resistance to chemotherapy and molecular targeted therapies is a major hindrance to effective therapy, and cancer drug resistance remains an inevitable consequence of the majority of cases (Baldo and Pham 2013; Horne, Stevens et al. 2013).

In the current era of precision medicine, much effort has been placed on the identification and targeting of cancer-specific molecular markers with the aim to maximize tumor cell death while minimizing toxicity to normal, healthy cells. The hypothesis behind this strategy seems very solid. Cancer is understood by many as the result of a number of specific gene mutations. If these mutations could be targeted, cancer cells could then be effectively eliminated. Major advances in technology including high-throughput genome sequencing and microarray analyses have been employed to identify many candidate molecular targets in cancers, with the hope of pinpointing key pathways by which tumor cells require in order to thrive, providing Achilles's heels that could be targeted to eradicate cancer (Horne, Stevens et al. 2013).

This strategy of targeting "oncogene addiction" has been largely influenced by one of the great success stories in molecular medicine, the targeting of chronic myeloid leukemia patients in the chronic phase with imatinib. The molecular characterization of chronic myeloid leukemia revealed a translocation of chromosomes 9 and 23 (now commonly referred to as the "Philadelphia chromosome"), which results in the de novo formation of the Bcr-Abl fusion oncogene, a constitutively active form of the Abl tyrosine kinase

(Nowell and Hungerford 1960; Rowley 1973). Bcr-Abl kinase hyperactivity is associated with enhanced proliferation and growth factor independence while reducing apoptosis (Jabbour, Hochhaus et al. 2010; Zhang and Rowley 2011). Furthermore, in the vast majority of chronic myeloid leukemia patients in the chronic phase, the Philadelphia chromosome (and thus the presence of the Bcr-Abl fusion gene) represents the sole chromosome aberration observed in leukemia cells (Johansson, Fioretos et al. 2002). The mutant Bcr-Abl kinase represented a specific cancer target not observed within normal, healthy somatic cells, and it was proposed that if this gene could be targeted, the cancer could be cured. Imatinib was developed to inhibit the activity of the Bcr-Abl kinase by blocking the ATP-binding site, suppressing kinase signaling and inducing cell death (Druker, Tamura et al. 1996; Druker, Sawyers et al. 2001). Results have been impressive for chronic myeloid leukemia patients in the chronic phase, with a seven-year overall survival rate of 86% (Jabbour, Hochhaus et al. 2010), and imatinib is currently accepted as the standard of care for chronic phase patients (Horne, Stevens et al. 2013).

Unfortunately, this overwhelming success has yet to be repeated for the vast majority of solid tumors, which represent 90% of all malignancies (Heng, Liu et al. 2010; Horne, Stevens et al. 2013). Most solid tumors do not follow the clonal, stepwise expansion model observed in select stages of hematological cancers such as the chronic phase of chronic myeloid leukemia (Johansson, Fioretos et al. 2002). Rather, cancer progression of most solid tumors consists

of both highly dynamic, stochastic phases as well as periods of stepwise, Darwinian progression (Heng, Stevens et al. 2006; Navin, Kendall et al. 2011; Heng 2015; Horne, Ye et al. 2015a). Most solid tumors are also marked by high degrees of genome heterogeneity, where even tumors of the same type will often display unique mutations and karyotypes (Heppner and Miller 1998; Heng, Stevens et al. 2004; Heng, Stevens et al. 2006; Losi, Baisse et al. 2005; Merlo, Pepper et al. 2006). This heterogeneity has been further confirmed using high-throughput DNA sequencing (Gerlinger, Rowan et al. 2012). Realization of these key evolutionary and cell population differences between exceptional hematological cases and the vast majority of solid tumors was recently accomplished with application of the genome theory of cancer evolution, solving the puzzle regarding the shortcomings of targeting cancer therapy for the majority of cases (Horne, Stevens et al. 2013).

Even for chronic myeloid leukemia, when heterogeneity is high (especially at the genome level), the power of targeting therapy is lost. The efficacy of imatinib sharply declines as chronic myeloid leukemia progresses from the more homogenous chronic phase to the highly heterogeneous “blast crisis” stage. Complete cytogenetic response in early chronic phase patients placed on imatinib is approximately 80%, and this falls to approximately 8% in the blast crisis stage (Radich 2007). This advanced stage resembles the majority of solid tumors, displaying increased genomic instability and genetic changes at multiple levels (Horne, Stevens et al. 2013). The frequency of additional chromosomal

abnormalities beyond the Philadelphia chromosome is approximately 7% in chronic phase patients and jumps to 40-70% in later stages (Skorski 2011). The median survival time of patients in blast crisis is measured in months (Assouline and Lipton 2011). It is worth noting that the linkage between genomic instability and poor prognosis has been documented in both hematologic and solid cancer patients (Nishizaki, Harada et al. 2002; Nakamura, Saji et al. 2003; Caraway, Thomas et al. 2008; Sato, Uzawa et al. 2010; Zamecnikova, Al Bahar et al. 2010). This complicates the treatment of most cancers further, because the heterogeneity observed at multiple levels is associated with drug resistance.

Current molecular understandings of cancer drug resistance

Several molecular mechanisms have been associated with drug resistance against the wide array of general chemotherapeutic and specific-targeting agents administered to patients (Table 1). These mechanisms range widely and include mutations of a drug target, increased expression of a drug target, activation of DNA repair mechanisms, alterations of drug metabolism, inactivation of cell death pathways, activation of survival signaling pathways, increased rates of drug efflux through activation and overexpression of membrane-bound transporter proteins, epigenetic mechanisms such as the inactivation of genes through methylation that are essential to the conversion of the inactive administered drugs to their active state, amplification of a drug target, downregulation or mutations of enzymes involved in drug inactivating metabolic pathways, and activation of alternative signaling pathways (Saunders, Simpson

Table 1: Overview of select cancer drug resistance mechanisms to commonly administered chemotherapeutic and specific-targeting agents

Therapeutic Agent/Drug Class	General or Targeting Agent	Cancer Type(s)	Drug Target	Mechanism(s) of Resistance	References
Antimetabolites (e.g. 5-fluorouracil, methotrexate)	General	Lymphoma, leukemia, ovarian cancer, breast cancer, colorectal cancer, pancreatic cancer, gastric cancer, head and neck cancer	Thymidylate synthase, DNA synthesis	Elevated thymidylate synthase expression, activation of survival pathways (e.g. ERBB signaling pathways), MLH1 hypermethylation, elevated expression of anti-apoptotic proteins	(Miyashita and Reed 1992; Johnston, Lenz et al. 1995; Brown, Hirst et al. 1997; Chen, Dai et al. 2007; Hurwitz, Stasik et al. 2012)
Bevacizumab	Targeting	Non-small cell lung cancer, colorectal cancer, renal cell carcinoma, glioblastoma	VEGF	Alternative signaling pathways activation (e.g. IGF1R, PDGFR), tumor dormancy induction	(Piao, Liang et al. 2012; Jahangiri, De Lay et al. 2013)
Bortezomib	Targeting	Mantle cell lymphoma and multiple myeloma	Proteasome	Anti-apoptotic mechanisms, bortezomib binding site mutations	(Oerlemans, Franke et al. 2008; Busacca, Chacko et al. 2013)
Cetuximab	Targeting	Colorectal cancer and head and neck cancer	EGFR	KRAS mutation, cetuximab binding inhibited by EGFR-S492R mutation	(Lièvre, Bachet et al. 2006; Montagut, Dalmases et al. 2012)
Crizotinib	Targeting	Non-small cell lung cancer	EML4-ALK	CD74-ROS1 rearrangement, secondary EML4-ALK mutation or rearrangment	(Shaw, Yeap et al. 2011; Bergethon, Shaw et al. 2012; Camidge, Bang et al. 2012)
Gefitinib	Targeting	Non-small cell lung cancer	EGFR	Epigenetic mechanisms, elevated ERBB family signaling	(Van Schaeybroeck, Karaiskou-McCaul et al. 2005; Li, Wu et al. 2013)
Imatinib	Targeting	Chronic myeloid leukemia	BCR-ABL	Target mutations	(Shah, Tran et al. 2004; Deininger,

					Buchdunger et al. 2005)
Microtubule-targeted agents (e.g. docetaxel, paclitaxel)	General	Breast cancer, ovarian cancer, lung cancer, head and neck cancer	Tubulin	Mutations in tubulin, multi-drug resistance 1 transporter overexpression	(Giannakakou, Sackett et al. 1997; Duesberg, Stindl et al. 2000; Kavallaris, Tait et al. 2001; Thomas and Coley 2003; Swanton, Nicke et al. 2009)
Nilotinib	Targeting	Chronic myeloid leukemia	BCR-ABL	BCR-ABL upregulation	(Mahon, Hayette et al. 2008; Camgoz, Gencer et al. 2013)
Platinum agents (e.g. cisplatin)	General	Lymphoma, testicular cancer, ovarian cancer, sarcoma, small-cell lung carcinoma	DNA	Elevated DNA repair, reduced cellular uptake, elevated efflux, MLH1 hypermethylation	(Fink, Aebi et al. 1998; Thomas and Coley 2003; Usanova, Piée-Staffa et al. 2010)
Topoisomerase I and topoisomerase II inhibitors (e.g. doxorubicin, etoposide, irinotecan)	General	Colorectal cancer, leukemia, glioblastoma, small-cell lung carcinoma, lymphoma, Ewing's sarcoma, testicular cancer	Topoisomer-ase I and II	Multi-drug resistance 1 transporter overexpression, drug efflux, topoisomerase mutations, p53 mutation, reduced topoisomerase expression	(Sugimoto, Tsukahara et al. 1990; Bugg, Danks et al. 1991; Miyashita and Reed 1992; Thomas and Coley 2003)
Trastuzumab	Targeting	ERBB2-positive breast cancer	ERBB2	Alternative signaling pathway activation, loss of PTEN, ERBB2 mutation,	(Lu, Zi et al. 2001; Nagata, Lan et al. 2004; Recupero, Daniele et al. 2013)
Vemurafenib	Targeting	Melanoma	BRAF-V600E	KRAS, MEK1, NRAS mutations	(Nazarian, Shi et al. 2010; Wagle, Emery et al. 2011)

et al. 2012; Holohan, Van Schaeybroeck et al. 2013; Zahreddine, Borden et al. 2013; Hu and Zhang 2016).

Immunotherapy is an emerging therapeutic approach that has gained excitement through early successes of inducing long-term tumor regression (Restifo, Dudley et al. 2012; Restifo, Smyth et al. 2016). The strategy behind immunotherapy involves the transfer of either gene-engineered or naturally occurring T-cells that target specific antigens expressed by tumor cells to patients. Despite early promise, there are examples of proposed resistance mechanisms to immunotherapy (Restifo, Smyth et al. 2016). This includes the complete loss of β_2 microglobulin observed in patient tumor samples. β_2 microglobulin is a component of major histocompatibility complex class I molecules, which present peptides to the therapeutic CD8+ T-cells.

Despite many successes of various therapies based on these varying molecular mechanisms (each showing promising results in in vitro studies and in many animal models, as well as improved response in some patients), with few exceptions, drug resistance universally occurs rather rapidly. This is even the case for current, very promising immunotherapy, indicating an important mission to search for the most common mechanism of drug resistance beyond diverse molecular mechanisms.

As discussed, the intended result of the administration of high-dose general and specific-targeting therapeutics is the maximal induction of tumor cell death. A survival strategy utilized by tumor cells was recently introduced that

occurs in response to high-level stress, and this strategy was termed “genome chaos” (Heng, Stevens et al. 2006; Heng, Liu et al. 2011; Liu, Stevens et al. 2014; Heng 2015). This process consists of rapid genome (i.e. karyotypic) fragmentation and re-organization, followed by the formation of chaotic genomes and the potential to establish new, stable genomes (i.e. different karyotypes), despite the initial massive cell death that comes as a cost to triggering genome chaos. Chromosome fragmentation and genome chaos have been induced by various stresses, including loss of gene function (e.g. ATM, ATR, p53), oxidative stress, temperature change and different types of drug treatment at high-dose concentrations (e.g. docetaxel, doxorubicin, methotrexate, mitomycin-C) (Stevens, Liu et al. 2007; Stevens, Abdallah et al. 2011; Liu, Stevens et al. 2014). The terminology of “karyotypic chaos” and “chromosome chaos” were initially used to describe these extensive chromosomal changes in addition to genome chaos (Heng, Stevens et al. 2006; Duesberg 2007; Heng 2007; Heng, Liu et al. 2011).

The logical question for us was, could such genome evolutionary-based mechanism serve as the framework to explain the ultimate failure that is cancer drug resistance? However, this idea was largely ignored by research communities, due to gene mutation-centric cancer research. For decades, genome level alteration (e.g. rapid genome re-organization induced by drug treatment) has been considered by most researchers as insignificant artifacts, as

it was impossible to imagine these massively altered genomes as survivable (Heng 2015).

Genome chaos has recently become a hot topic in cancer research, because chaotic genomes have been confirmed as a result of the cancer genome sequencing project using advanced high-throughput DNA sequencing of numerous clinical samples. They are no longer considered as in vitro artifacts because these can be detected in most cancer patients. Furthermore, they are also detected in normal tissue types, earlier developmental stages, as well as other diseases (Ye, Liu et al. 2007; Iourov, Vorsanova et al. 2008; Celton-Morizur and Desdouets 2010; Davoli and de Lange 2011; Fragouli and Wells 2011; Iourov, Vorsanova et al. 2012a; Iourov, Vorsanova et al. 2012b; Heng, Liu et al. 2013; Hojsak, Gagro et al. 2013; Hultén, Jonasson et al. 2013; Horne and Heng 2014).

Many new terms have been introduced to describe chromosome fragmentation and re-organization, including “chromothripsis,” “chromoplexy,” “chromoanagenesis,” “chromoanasynthesis,” “chromosome catastrophes,” and “structural mutations” (Liu, Erez et al. 2011; Meyerson and Pellman 2011; Stephens, Greenman et al. 2011; Tubio and Estivill 2011; Crasta, Ganem et al. 2012; Forment, Kaidi et al. 2012; Holland and Cleveland 2012; Inaki and Liu 2012; Jones and Jallepalli 2012; Righolt and Mai 2012; Setlur and Lee 2012; Baca, Prandi et al. 2013; Malhotra, Lindberg et al. 2013). Furthermore, rapidly generated re-organized genomes have been detected within various types of

cancer, and these chaotic genomes have been displayed in the majority of cases of some cancer types (Heng, Liu et al. 2011; Heng, Stevens et al. 2011; Stephens, Greenman et al. 2011; Baca, Prandi et al. 2013). Understanding the importance of genome chaos as it pertains to cancer drug resistance would be realized in the context of genome-mediated cancer evolution (Liu, Stevens et al. 2014; Heng 2015).

Macro-cellular evolution mediated genome heterogeneity plays a critical role in cancer drug resistance

The two phases of cancer evolution were originally based on the karyotypic pattern observed in an immortalization model where both clonal and non-clonal expansions were detected (Heng, Stevens et al. 2006). These were recently confirmed in breast cancer using single cell level genome sequencing (Navin, Kendall et al. 2011; Wang, Waters et al. 2014). Cancer evolution is a series of genome-mediated system replacements occurring in dynamic cycles of non-clonal chromosome aberrations (NCCAs) and clonal chromosome aberrations (CCAs) within the two evolutionary phases (Figure 1). In the stepwise phase, the majority of cells are clonal across generations, and karyotypic diversification is traceable. The punctuated phase is characterized with a high frequency of NCCAs and massive genome reorganization, which break multiple system constraints (e.g. genome integrity, tissue architecture, etc.). Thus, cancer progression consists of both macro-cellular (genome system replacement) and micro-cellular (modification and diversification of the genome-defined system) evolution. There is recent increased support for the macro-

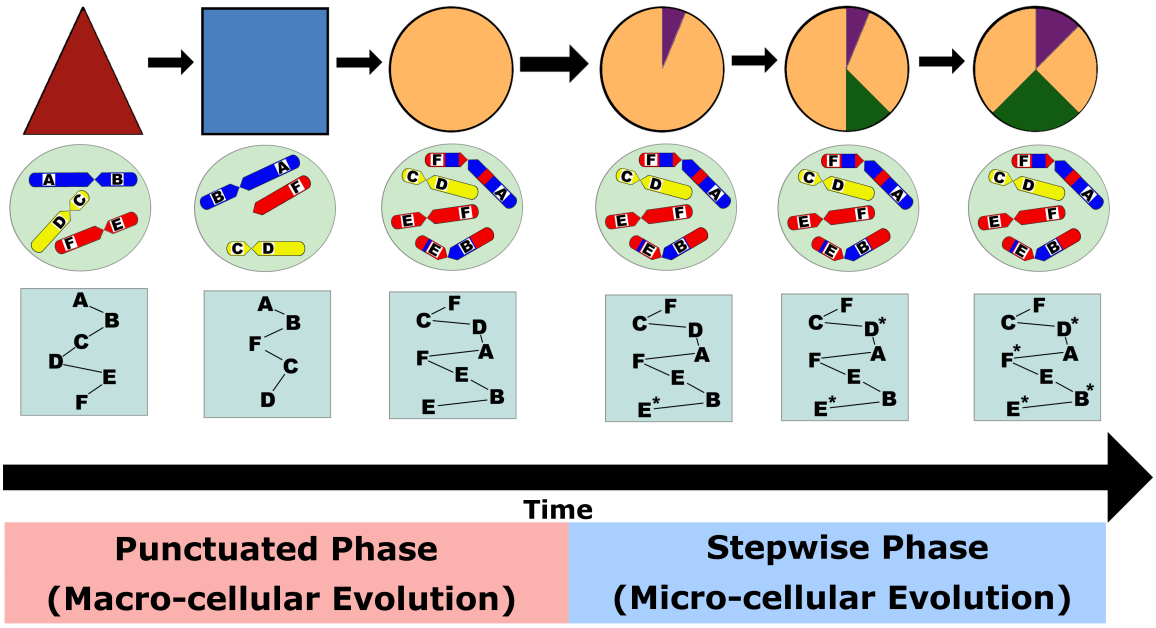


Figure 1. Stochastic model of genome-mediated cancer evolution. Cancer evolution is divided into two distinct evolutionary phases, the punctuated stochastic phase (or macro-evolutionary phase) and the stepwise gradual phase (or micro-evolutionary phase). Punctuated phases are marked by extreme heterogeneity and rapid genome changes, represented by genome system changes over time, with each shape representing a unique genome system. Different chromosomes are designated by color (red, yellow, blue) and drawn within the nucleus below the corresponding system. Genes are designated A, B, C, D, E, F within the chromosomes, and corresponding protein networks are illustrated below by the relationships between proteins A, B, C, D, E, F. The punctuated phase is caused by system instability-mediated macro-cellular evolution, resulting in high NCCA frequency illustrated by different genome systems (shapes), topologies (karyotypes, including numerical and/or structural chromosome aberrations), and protein interactive networks. Following selection pressure, a unique genome system survives (circle). In contrast to genomes in the punctuated phase, this genome system in the stepwise phase remains relatively stable over time, although it does acquire low-level changes (represented by pie piece changes) such as gene mutations, epigenetic alterations and/or small traceable genome-level alterations that aide in adaptation. Genetic/epigenetic alteration is indicated by asterisks (*) in the protein network. These micro-cellular changes can be classified into clonal expansion and diversification. Thus, the stepwise phase is mainly associated with system stability and micro-cellular evolution. Only one run of the NCCA/CCA cycle is presented. Figure reproduced from Horne, Wexler et al. 2015 with permission from http://AtlasGeneticsOncology.org.

micro phases of evolution in cancer concept (Klein 2013).

The importance of genome chaos in cancer evolution was realized with the incorporation of the concept of genome topology-defined system inheritance. Under the genome theory of cancer evolution, which has been developed in the laboratory of Dr. Henry Heng for over a decade, new karyotypes define new system inheritance, as similar gene content can result in different inheritance by altering the interactive relationships of genes or three-dimensional genomic topology (Heng, Stevens et al. 2006; Heng 2009; Heng, Stevens et al. 2010; Heng, Liu et al. 2011; Horne, Ye et al. 2015a). In organismal evolution, the main function of sexual reproduction is to preserve the karyotype (or species-specific system), thus preserving system inheritance (Wilkins and Holliday 2009; Gorelick and Heng 2011; Heng, Liu et al. 2011). This differs from cancer, where the cancer genome needs to change to form new systems for a shot at survival, as drastically altered chromosome structure functions as a systems oncogenic organizer (Inaki and Liu 2012). Further, genome chaos and genome instability have been linked with elevated transcriptome dynamics and increased evolutionary potential (Stevens, Liu et al. 2014). To summarize, as a result of drug-induced genome chaos, genome heterogeneity is drastically increased through the rapid formation of new genome systems. This gives cancer the opportunity to survive, as some of these new systems are fit to survive the crisis event (e.g. drug treatment) and clonally expand. Considering the stochastic nature of generating new systems through genome chaos, this can explain the

many different molecular mechanisms discussed that have been identified in cancer cells and previously associated with cancer drug resistance. Clonal expansion during the stepwise phase (after drug treatment and selection) may result in a cell population expressing a particular gene or pathway that is easily detectable. However, considering that cancer evolution is a highly dynamic process that includes new system formation, that gene or pathway does not reflect on the overall process of drug resistance, and the role of a particular gene or pathway may change over time, between patients, and may differ from cell to cell.

It should be pointed out that the theoretical thinking that linked the karyotype as a new layer of genetic coding (or blueprint) and that altered genomes represent new biosystems with new network structures is essential to understanding genome-mediated drug resistance. It provides insight to how massive genome changes promote the formation of new systems during cancer drug resistance. If these dynamic, drastically changing identities are emergent new systems, it is no wonder that some of these could become dominant in the face of the high selective pressure of high-dose therapeutics. The next question is, what is the detailed picture of the outliers being selected when there are so many cells with different genomes?

Recent study of heterogeneous cell populations demonstrated the power of outliers in terms of cancer progression and emergent features such as growth (Abdallah, Horne et al. 2013). Using single-cell and population-based assays of

karyotypically stable and unstable cells, it was shown that, within a heterogeneous cell population, outliers dominantly contributed to overall cell population growth through rapid proliferation. These findings can be extended to the importance of drug-induced individual chaotic genomes in cancer drug resistance. While unstable, these outlier genomes can rapidly evolve until stable clones emerge and thrive, representing the only chance for survival under aggressive drug treatment. While the majority of cells may be eliminated through high-dose therapeutic regimens, the formation of aggressive outlier subgroups through genome chaos may be sufficient to drive cancer progression post-treatment and rapidly generate lost tumor cell counts. We explore this further in Chapter 2.

The adaptive function of stress-induced genome alteration

Genome alteration has adaptive function under stress. We previously introduced an evolutionary trade-off between stress, genomic adaptation and the onset of common diseases (Horne, Chowdhury et al. 2014), providing a basis for the many common diseases that lack a clear, causative molecular linkage or heritable factor. High-level genome alterations and elevated genome instability have been reported in a wide variety of common diseases. These include Alzheimer's disease, autism, Gulf War illness, Crohn's disease and chronic fatigue syndrome (Ye, Liu et al. 2007; Iourov, Vorsanova et al. 2008; Iourov, Vorsanova et al. 2012a; Iourov, Vorsanova et al. 2012b; Heng, Liu et al. 2013; Hojsak, Gagro et al. 2013; Heng, Horne et al. 2016). Surprisingly, genome

alterations have also been observed in various normal, healthy tissues, which include the polyploidization of liver cells, skeletal muscle, placenta, ovary, thyroid gland, urothelium, blood, blastocyst mosaicism, Purkinje neurons, trisomy 21 mosaicism in the general population, as well as detected stochastic karyotypic changes as the result of environmental and physiological challenges (Biesterfeld, Gerres et al. 1994; Heng, Stevens et al. 2004; Celton-Morizur, Desdouets et al. 2010; Davoli and de Lange 2011; Fragouli and Wells 2011; Hultén, Jonasson et al. 2013). In addition, recent whole genome sequencing of healthy individuals revealed increased genome-level alteration (1000 Genomes Project Consortium, Abecasis et al. 2012). It is understood that cells at any given time are subject to various internal and external stresses, under either normal physiological or pathological conditions. Stress, in general, results in many infrequent genome alterations (Heng, Stevens et al. 2004; Heng, Stevens et al. 2006). Genome-level alterations are more effective at drastically changing the genetic system than gene mutation or epigenetic change. This suggests that stress-induced genome level change could effectively provide an adaptive advantage for cells against high levels of environmental stress. Further, genome diversity within normal, healthy tissues allows for complex organ function while providing the genome heterogeneity (or robustness) necessary to account for organ-function associated stress (e.g. liver-mediated blood detoxification). We concluded that stress-induced heterogeneity is necessary for successful adaptation to occur, but the trade-off is potential disease onset (Horne, Chowdhury et al. 2014; Horne,

Pollick et al. 2015; Heng 2015; Heng, Horne et al. 2016). Taking into account the new function of sexual reproduction as a constraint and filter to eliminate large-scale genome aberrations from the germline, we understood how system dynamics are promoted for short-term adaptation at the individual level, while the accumulation and passing of alterations to offspring is prevented, and this realization provided clarification behind the "missing inheritability" of many common diseases (Heng 2010).

The majority of cancer drug resistance research and discussion has neglected the macro-cellular evolutionary phase of cancer

Cancer drug resistance is often regarded in two categories: intrinsic or acquired. Intrinsic resistance would suggest that pre-existing factors (e.g. increased drug efflux, mutations of drug targets) involved in drug resistance are present within cells prior to treatment. Upon treatment, tumor cells sensitive to the treatment would be eliminated, and those that are resistant continue to thrive. Acquired cancer drug resistance is considered as drug resistance that develops during treatment of tumor cells that were initially sensitive, and this can be the result of mutations of drug targets as well as through other adaptive responses such as increased expression of therapeutic targets, activation of alternative compensatory signaling pathways, epigenetic changes, activation of survival signaling pathways, activation of DNA repair processes, inactivation of downstream cell death signaling pathways, alterations of drug metabolism and increased rates of drug efflux (Saunders, Simpson et al. 2012; Holohan, Van Schaeybroeck et al. 2013; Zahreddine and Borden 2013; Hu and Zhang 2016).

From an evolutionary perspective, most cancer drug resistance study and discussion pertains to the micro-evolutionary phase. This is due to the emphasis made on lower level alterations (e.g. gene mutations, epigenetic changes, expression changes) that are proposed to either be selected for upon treatment or emerge during treatment in a stepwise, gradual manner resulting in cancer drug resistance. However, macro-cellular evolution plays a major role in cancer drug resistance through the formation of new genome systems that survive therapy and recovery. Considering that this highly dynamic phase can be triggered by high-dose therapeutics, which represents the current standard of care, problems regarding cancer drug resistance will not be solved without accounting for macro-cellular cancer evolution. While lower level genetic change can potentially be useful in understanding exceptional cancer cases, notably those of high karyotypic homogeneity (e.g. Bcr-Abl fusion gene positive chronic myeloid leukemia patients in the chronic phase, Pml-Rara fusion gene positive acute promyelocytic leukemia patients) as well as physiological conditions, it falls short in providing clinically relevant explanations for cases where genome heterogeneity is high (Horne, Stevens et al. 2013). Unfortunately, this is the situation for the vast majority of cancers, as these are marked by high degrees of intra- and inter-tumor genome heterogeneity at multiple genetic and non-genetic levels (Heppner 1984; Heng, Bremer et al. 2009).

Overview

Here, cancer drug resistance is elucidated through application of macro-cellular cancer evolutionary theory, which should explain the common failure of aggressively treating against diverse molecular mechanisms and provide new thinking for how to deal with this key challenge. In Chapter 2, using experimentation and analysis designed to account for both heterogeneity (i.e. genome, cell growth) and monitor long-term system behavior, we identify a general adaptive mechanism of cancer drug resistance that is triggered by high-dose therapeutics. This is a transition that illustrates a key clinical paradox between initial effective tumor cell killing with high-dose treatment and robust, long-term cancer drug resistance. It is comprised of the following: first, drug-induced genome chaos (rapid genome fragmentation and reorganization); second, increased genome heterogeneity, the necessary pre-condition for the formation of aggressive outliers; and third, aggressive outlier-driven progression, represented by the emergence of new features such as rapid proliferation. In Chapter 3, we search for a conceptual framework to accurately study the pattern of cancer evolution, which accounts for both heterogeneity and outlier contributions rather than focusing on specific patterns and initial killing impact. This analysis includes defining key parameters for studying heterogeneous populations under stress, prioritizing genetic levels to construct accurate understandings of complex diseases including cancer, shifting research focus to single cell resolution and classifying biomedical research studies to aide in

selecting informative analytical approaches. From our synthesis in Chapter 3, proper conceptual framework is necessary in research as different analytical approaches lead to drastically different conclusions. Such analysis is timely to the field of molecular cancer research, as there is a heated debate about the reliability of data. This analysis will provide insight and guidance for experimental design and data collection for highly heterogeneous systems. Finally, we provide conclusions and future directions in Chapter 4. These include the call for in-depth profiling and characterization of stress-induced aggressive outliers involved in cancer drug resistance (e.g. metabolics, phenotypic features beyond growth patterns), determination of therapeutic thresholds that trigger genome chaos, and points to consider from this project as they relate to current large-scale efforts geared towards the curing of cancer, such as the Moon Shots Program.

CHAPTER 2: EFFECTIVE DRUG TREATMENT INDUCES DRUG RESISTANCE THROUGH RAPID GENOME ALTERATION-MEDIATED CANCER EVOLUTION

Introduction

Cancer patients are typically treated at or near the maximum tolerated dose of cytotoxic drugs with the implicit goal to eliminate tumor cells at maximal rate (Marshall 2012). Tumor cell response is often transient, and therapy fails with rare exceptions due to the emergence of drug resistant populations. The unfortunate clinical situation is, as therapy becomes more and more effective, acquired resistance also becomes more common (Gottesman 2002). Interestingly, mathematical and evolutionary modeling predicted that therapeutic intervention could provide selective pressure for the expansion of resistant variants (Maley, Reid et al. 2004; Pepper 2012), and initial drug response has been noted as “not a strong predictor of reduced mortality” (Pepper 2011). These models confirm a paradox of current cancer therapeutic strategies, where initially effective treatment, which eliminates a large number of tumor cells, also favors the formation of resistant clones. Such paradox significantly contributes to the key gap between treatment response (i.e. initial killing power) and patient survival. One evolutionary explanation has been that tumor cells are highly heterogeneous, and while effective initial treatment can wipe out the clonal population, other subpopulations including some cancer stem cells can take over, especially when drug treatment eliminates the competition (i.e. drug-sensitive tumor cells) of intrinsically resistant tumor cells for space and resources. This

would provide the perfect opportunity for resistant cells to rapidly overgrow (Gatenby, Silva et al. 2009; Saunders, Simpson et al. 2012). The specific mechanism of how treatment promotes resistance, however, has been unclear. Given the ultimate importance of this issue and the fact that rapid, developed drug resistance is common both in animal models and especially in the majority of patients, *in vitro* models are needed to dissect the entire evolutionary process of cancer drug resistance (from prior to treatment to weeks following administration) and to pinpoint the general mechanism.

A recently illustrated relationship between high stress and macro-cellular cancer evolution opened a new avenue for studying a general mechanism of drug resistance (Liu, Stevens et al. 2014; Horne, Ye et al. 2015a; Horne, Ye et al. 2015b). Drug treatment has been linked to genome chaos, a phenomenon characterized by rapid and massive genome reorganization induced by external and internal stresses (Heng, Stevens et al. 2006; Duesberg 2007; Heng 2007; Heng, Liu et al. 2011; Heng, Stevens et al. 2011; Liu, Stevens et al. 2014). Genome chaos has also been linked to elevated transcriptome dynamics (Stevens, Horne et al. 2013; Stevens, Liu et al. 2014), and some chaotic genomes with increased evolutionary potential (reflected by increased transcriptome dynamics) could function as “lucky outliers” and dominate a cancer cell population with new features such as increased cell growth (Abdallah, Horne et al. 2013).

We thus hypothesized that the domination of outliers from drug-induced genome chaos might be the common mechanism of rapid drug resistance in cancer. To test this hypothesis, we needed to illustrate the following: 1) high-dose treatment eliminates more cells initially but also induces increased genome chaos; 2) induced high levels of heterogeneity and increased outliers, in particular, are responsible for the paradoxical transition from initial high cell death to robust drug resistance; and 3) surviving clones display altered genomes resulting from genome chaos, which differ from those displayed in populations prior to treatment. To achieve this goal, in contrast to general approaches that mainly focus on cell death immediately following drug treatment, we follow the cell population dynamics for weeks until resistant clones become dominant. Overall growth, population diversity, survival rate and genome profiles have been recorded to understand the mechanism of drug resistance under high-dose treatment.

Methods and Materials

Cell lines and cell culture

HCT116 cell line was obtained from ATCC. HCT116 cells were maintained in high-glucose DMEM media (Gibco), supplemented with 10% FBS (Gibco) and 1% Penicillin-Streptomycin solution (Hyclone). Cell culture flasks and plates were incubated at 37^{o}C and 5% CO_2. The cell line was checked and authenticated using spectral karyotyping methods.

In situ doxorubicin cell population treatment and long-term culture counting

Four hundred (400) HCT116 cells were plated in each of three culture flasks. Immediately after plating, doxorubicin treatments (0, 50, 100nmol/L) were added to the flasks. Treatments lasted 16 hours, and media was then replaced with fresh media. Cells were maintained in culture for an additional 20 days, including media changes every 24-48 hours and culture splitting by trypsinization and dilution when cell culture confluence approached 70-80%. Cell cultures from each flask were then trypsinized, and equal aliquots of cells were added into sets of 3-4 flasks. Cells from a flask from each set would be trypsinized and counted using hemacytometer methods; one flask from each set would be counted per day for 3-4 days. Doubling times from each set would be calculated based on the daily cell totals, and those would be used to calculate overall cell totals for each treatment condition. After trypsinization and hemacytometer counting, equal aliquots of the cell cultures from the final flask of each set would be added in sets of 3-4 flasks, and this long-term cell culture and counting process would continue for a total of 85 days.

In situ doxorubicin cell population treatment and post-recovery counting

For each trial (of nine), 5000 HCT116 cells were plated per well of six-well culture plates, totaling ten treatment and four untreated control samples per trial. In six of these trials, cells were plated for 24 hours before treated with 1.0ug/ml doxorubicin for two hours to induce genome chaos. In the remaining three trials, cells were plated for 24 hours before treated with 0.1ug/ml doxorubicin for two hours as a low-dose regimen serving as validation of the high-dose trials.

Untreated control cells in all trials were treated with sterile water (doxorubicin vehicle). Treatment was immediately followed by a brief 1x PBS wash and replaced with fresh media. Cells remained in culture for 24 days to recover. Cells from each well were then trypsinized, and using hemacytometer methods, four aliquots of 1,000 cells from each sample were individually plated in wells of new 24-well plates and grown in culture for seven days. Cell totals were then counted using hemacytometer methods and average totals were compared to untreated HCT116 control average totals.

In situ doxorubicin treatment and post-recovery single cell-derived population counting

HCT116 cells grown in a T-25 culture flask were treated with 1.0ug/ml doxorubicin for two hours. Treatment was immediately followed by a brief 1x PBS wash and replaced with fresh media. After recovery, cells were released from the flask by mitotic shake-off, and using hemacytometer methods, 400 cells were plated in a new T-25 flask, labeled with grids. Twenty single cells were identified, and growth was measured daily for five days. Cell totals were calculated for all single-cell derived subpopulations and compared to untreated HCT116 controls.

Multi-color chromosome painting and spectral karyotyping

To validate cell line identity and observe novel chromosome aberrations post-treatment, multi-color chromosome painting and spectral karyotyping were performed on mitotic spreads as previously described (Heng and Tsui 1993; Ye, Lu et al. 2001; Heng, Ye et al. 2003). Briefly, cytogenetic slides were prepared,

denatured and hybridized with human painting probes. After washing and spectral karyotyping detection, mitotic structures were captured using a charge coupled device camera.

Statistical analysis

Statistically significant differences between independent samples were evaluated nonparametrically using the Mann-Whitney-Wilcoxon test. Degrees of cell total heterogeneity within different treatment conditions were determined through coefficient of variation calculations. Aggressive outliers were defined as outlier samples (greater than 1.5 times the interquartile range above the third quartile) that were also higher than all corresponding control samples. Statistical analyses were performed using R and Microsoft Excel software.

Results

The transition between initial, effective cell death and resistance was observed, yet at a low frequency

We followed survival and growth patterns of the human colon cancer cell line HCT116 after high-dose doxorubicin treatment. Genome chaos can be effectively induced under such treatment conditions (Liu, Stevens et al. 2014). This in vitro “watching evolution-in-action” experiment was designed to observe any potential transition between initial treatment-induced cell death and long-term growth after recovery (Figure 2). This approach holds an unique advantage over current in vivo models by providing a window of observation of cell populations over the entire treatment and recovery process and allows for precise extraction of cells for genetic/phenotypic analyses, whereas only end products (which may

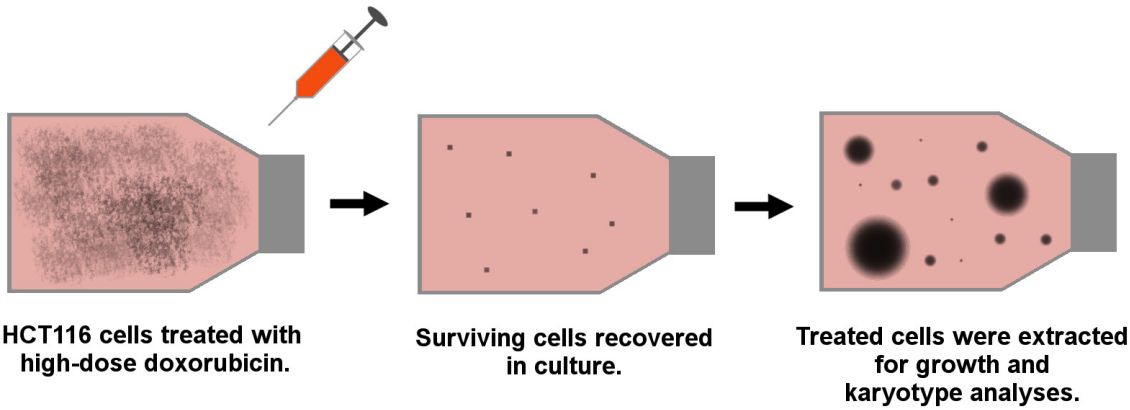


Figure 2: Schematic diagram of experimental design.

not be directly informative of the entire process) are extracted from many current in vivo models for analysis. Despite anticipated findings of high-dose treatment resulting in initial lower cell counts compared with low-dose and untreated control groups (eliminating ~90% of identified and traced single cells over seven days after treatment), a striking transition was observed. This was evidenced by recovered, high-dose treated cells outgrowing untreated cells after 31 days in culture (~18% higher tumor cell population total) (Figure 3). However, such exciting findings were difficult to replicate in order to demonstrate statistical significance. We failed to observe this transition between initial effective cell death and aggressive growth in two separate repeated trials. This raised the question: should we consider the observed transition a “false result” and disregard it? Despite its low frequency (1 in 3 attempts), it does fit the reality of cancer drug resistance. Equally important, the same level of uncertainty has been observed from time to time (Table 2), and it is well known that different patients display drastically different responses to treatments in terms of the onset of drug resistance. We realized that if the transition is real, the chance of inducing aggressive outlier formation, which could then dominate surviving cell population growth, must be very low. This low reproducibility might explain why such an important transition has been previously ignored. Taking this into consideration, we realized that we needed to illustrate: 1) regardless of whether treated outliers overgrow untreated control groups after recovery, the overall heterogeneity for all high dose groups should be increased following treatment

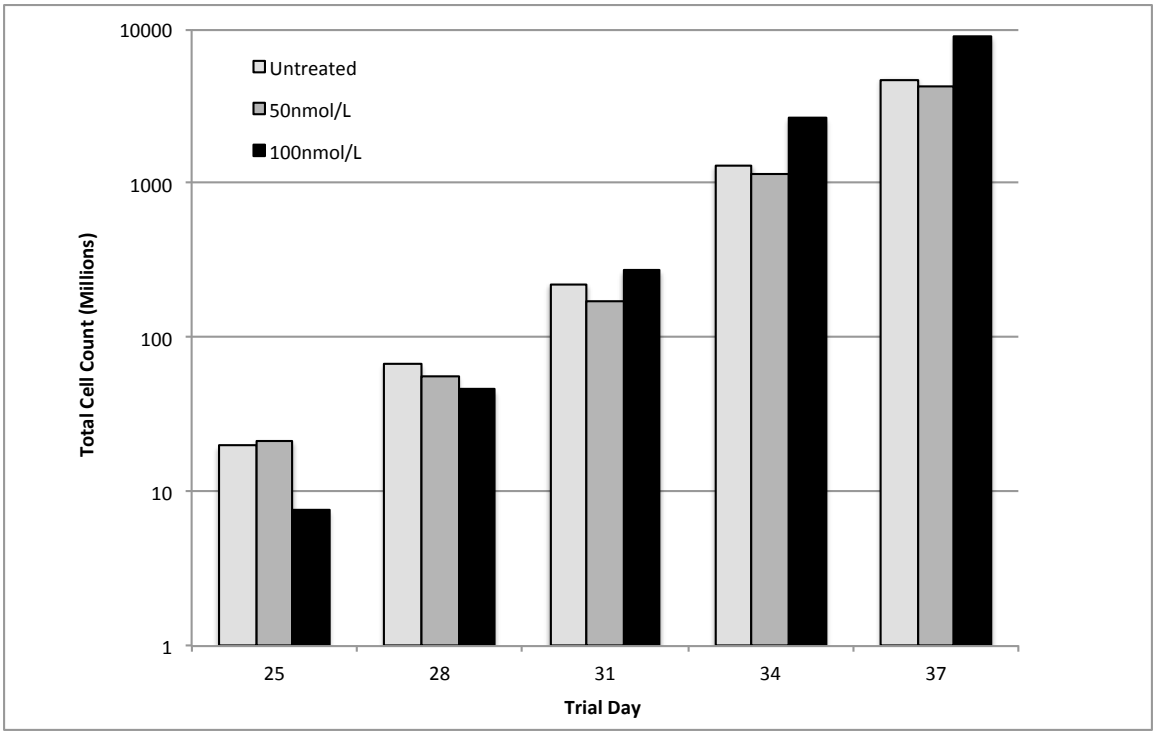


Figure 3: Recovered HCT116 cells display higher cell growth after high-dose treatment and long-term culture. Total HCT116 cell counts after doxorubicin treatment for 16 hours (0, 50, 100nmol/L) and recovery in long-term culture.

Table 2. Genome alteration observed across therapeutic approaches and cancer models.

Examples	References
Traditional drug resistant cell line selection Cells in vitro were treated with a panel of general chemotherapeutics (representing different mechanisms) of gradually increasing concentrations until resistant clones were selected. Resistant clones displayed altered karyotypes compared to cells prior to treatment.	Current study, Heng et al., unpublished observations
Cancer specific targeting Cells in vitro were treated with a small molecule inhibitor targeting cell death pathways. Clones that survived treatment displayed altered karyotypes.	Heng et al., unpublished observations
Genome chaos induction Genome chaos has been observed in vitro following various chemotherapeutics and linked to many diverse molecular phenomena and mechanisms.	Heng 2007; Duesberg 2007; Stevens, Liu et al. 2014; Liu, Stevens et al. 2014, Heng 2015; Current study
Patient sample sequencing following treatment Sequencing of matched pre- and post-treatment patient tumor cell samples revealed different genetic landscapes, including at the karyotype level.	Johnson, Mazor et al. 2014; Patch, Christie et al. 2015
Budding yeast variation following treatment Induced aneuploidy populations display increased phenotypic variation with increased treatment intensity, resulting in resistance.	Chen, Mulla et al. 2015

and recovery; 2) aggressive outliers that are products of genome chaos (of these, some will display fast growth) will emerge at low frequencies, and to demonstrate this transition, the likelihood must be examined quantitatively. Furthermore, since the process of genome-mediated drug resistance appears to be a stochastic process, a large number of duplicates are needed to test this hypothesis.

The real and rare event of successful, aggressive outlier formation was identified through quantitative analysis and requires drug treatment induced heterogeneity as a precondition

To quantitatively monitor population dynamics after treatment recovery, we systematically analyzed total cell growth of six separate, parallel trials, each consisting of ten treated samples and four untreated controls (Figure 4) (Appendix A). Since the same aggressive treatment conditions were used for all treated groups (previously established, clinically-relevant conditions for inducing genome chaos) (Liu, Stevens et al. 2014), initial high levels of cell death were observed across all 60 treated samples.

After recovery, the average growth of treated cells was lower than controls across all trials (Figure 4A), supporting the commonly accepted viewpoint that high-dose drug treatment slows down overall population recovery. We then examined whether growth heterogeneity was impacted as a result of treatment, and we observed a higher coefficient of variation in the treated samples compared to controls in all trials (Figure 4B). In other words, despite reduced numbers of survivors, the evolutionary potential of these treated populations is

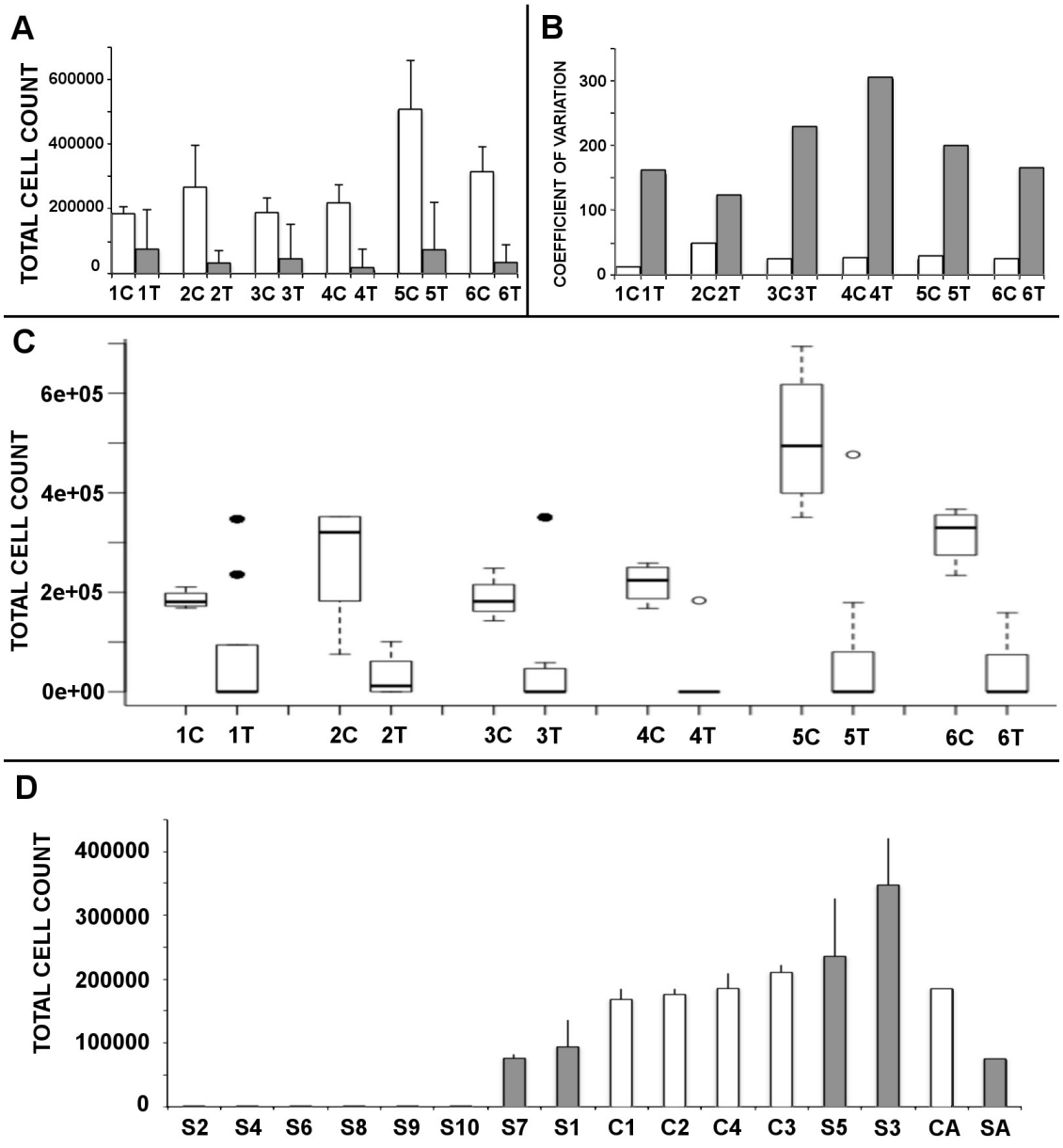


Figure 4. High-dose doxorubicin treatment results in decreased average cell population sizes, increased population growth heterogeneity and induced formation of aggressive outliers. **(A)** Total HCT116 cell counts post-treatment/recovery after seven days of growth. Each trial (of six) consists of treated samples (1T-6T) and untreated controls (1C-6C). Error bars represent S.D. **(B)** Coefficient of variation of treated samples and untreated controls. **(C)** Boxplots of total HCT116 cell counts of treated samples and untreated controls. Aggressive outliers (values greater than 1.5 times the interquartile range above

the third quartile and greater than corresponding control values) are labeled as solid black circles. Other outliers are labeled as empty circles. **(D)** Cell counts of individual samples (S1-S10) and untreated controls (C1-C4) from Trial 1 after seven days of growth. C1-C4 average (CA) and S1-S10 average (SA) are represented. Error bars represent S.D.

higher, as the degree of heterogeneity directly contributes to cancer evolution (Horne, Ye et al. 2015). It is important to note that higher variance does not necessarily translate to rapid resistance, as potential differs from reality. Most importantly, we confirmed the low probability event by identifying three aggressive outliers in Trials 1 and 3 that displayed higher growth than corresponding control samples (Figure 4C). In contrast, all control samples displayed lower variation for each trial than treated groups, as demonstrated by coefficient of variation calculations. As illustrated in Figure 4D, for one of the trials that displayed two aggressively growing samples, the remainder of treated samples much slower growth. In contrast, variation among control samples was low. This multiple, parallel trial (representing separate runs of evolution) analysis confirms the presence of a key transition in aggressive treatment resistance, as aggressive outlier formation is a rare but real event.

To validate these findings, we performed three additional trials with administration of a decreased concentration of doxorubicin by tenfold and compared these results with those of untreated and high-dose doxorubicin treated trials (Figure 5) (Appendix B). Significant differences were observed between the high-dose and untreated control groups in terms of average total cell counts and coefficients of variation ($p < 0.0004$) that were not observed between the untreated control and low-dose groups. This supports that increased growth heterogeneity (i.e. evolutionary potential) is the direct result of high-dose treatment.

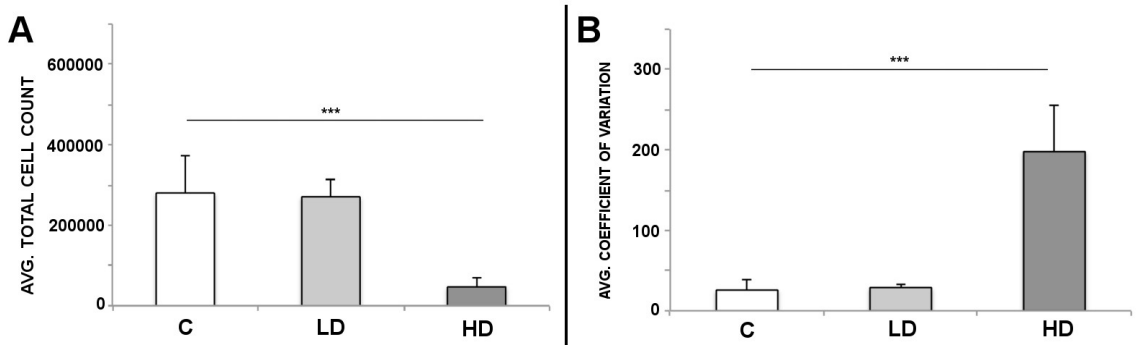


Figure 5. Low-dose doxorubicin treatment does not result in the significantly increased population growth heterogeneity observed after high-dose doxorubicin treatment. **(A)** Average total HCT116 cell counts post-treatment/recovery after seven days of growth for untreated controls (C), low-dose doxorubicin treated samples (LD), and high-dose doxorubicin treated samples (HD) of all trials. Error bars represent S.D. ***, $P < 0.0004$ (C vs. HD) **(B)** Average coefficient of variation of untreated controls (C), low-dose doxorubicin treated samples (LD), and high-dose doxorubicin treated samples (HD) of all trials. Error bars represent S.D. ***, $P < 0.0004$ (C vs. HD).

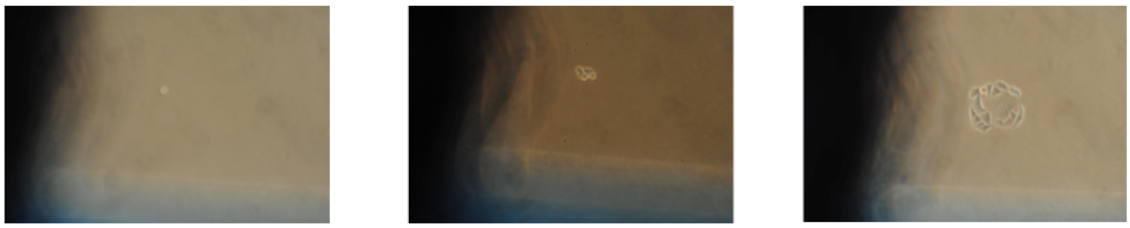
Additional factors that can increase the odds of induced drug resistance following initial treatment – further validation

In addition to initial dosage, what other factors can contribute to outlier success? According to the outlier behavior within a cell population, if the drug induced transition is a rare event driven by outliers, cell counts prior to drug treatment should be important, as more cells should generate more outliers. If this is true, it also validates our explanation. We performed additional trials using flasks of higher HCT116 cell confluence to maximize the opportunity of aggressive subgroup emergence (increasing the pre-treatment cell count per sample nearly 600-fold, from 5,000 to approximately 3,000,000 cells). Aggressive subgroups were consistently observed with these higher cell counts following drug treatment. While this transition is the result of minority events, a logical extension of these results to the many millions of tumor cells found in patients would suggest that this transition event is more of a certainty rather than an unlikely occurrence judged by a limited number of repeated experiments. It is worth noting that among many induced features that are essential for drug resistance, only a small portion of the surviving cell population displayed fast growth. Because we have only monitored the growth rates, important outliers are clearly underestimated. If multiple features are simultaneously monitored, more outliers should be identified. This fits into the clinical situation where resistance is the general rule rather than the exception, as somatic cell evolutionary selection acts on many different features with highly dynamic population replacement. Taking this added complexity into account, it is very likely that the

occurrence of successful outliers in the clinic is much higher, explaining why drug resistance is so common in the clinic.

Second, to demonstrate the contribution made by aggressive outliers to rapid drug resistance, in a separate trial, we performed single-cell derived population analysis of a recovered aggressive outlier population and compared to untreated controls (Figure 6) (Appendix C). Recovered subgroups on average displayed moderately, but not significantly, faster single-cell derived population growth than control subgroups ($p=0.0985$), and aggressive subgroups were also shown to be faster than all control subgroups (Figure 7A). This supports the concept that aggressive outlier populations that are the products of this process can dominate recovered populations and drive resistance through rapid proliferation.

Third, to confirm the role of genome alteration in this transition, we performed multi-color chromosome painting analysis to observe outlier karyotypes, and we confirmed that surviving treated cells displayed different genomes compared with untreated cell populations (Figure 7B). HCT116 serves as an excellent model analysis, as this cell line has remarkable documented karyotypic stability (Thompson, Compton et al. 2008; Knutsen, Padilla-Nash et al. 2010; Abdallah, Horne et al. 2013). Since all resistant clones display different karyotypes compared to the original karyotype of untreated populations, and it is well-characterized that genome chaos represents a necessary process to generate new, genome-defined systems (Liu, Stevens et al. 2014). It is worth



Day 1 **Day 3** **Day 5**

Figure 6. Single-cell derived population growth analysis. Cells were plated in T-25 flasks labeled with grids at a concentration of 400 cells/flask. On Day 1, twenty single cells per flask were identified and recorded. Growth was monitored under light microscopy and recorded daily.

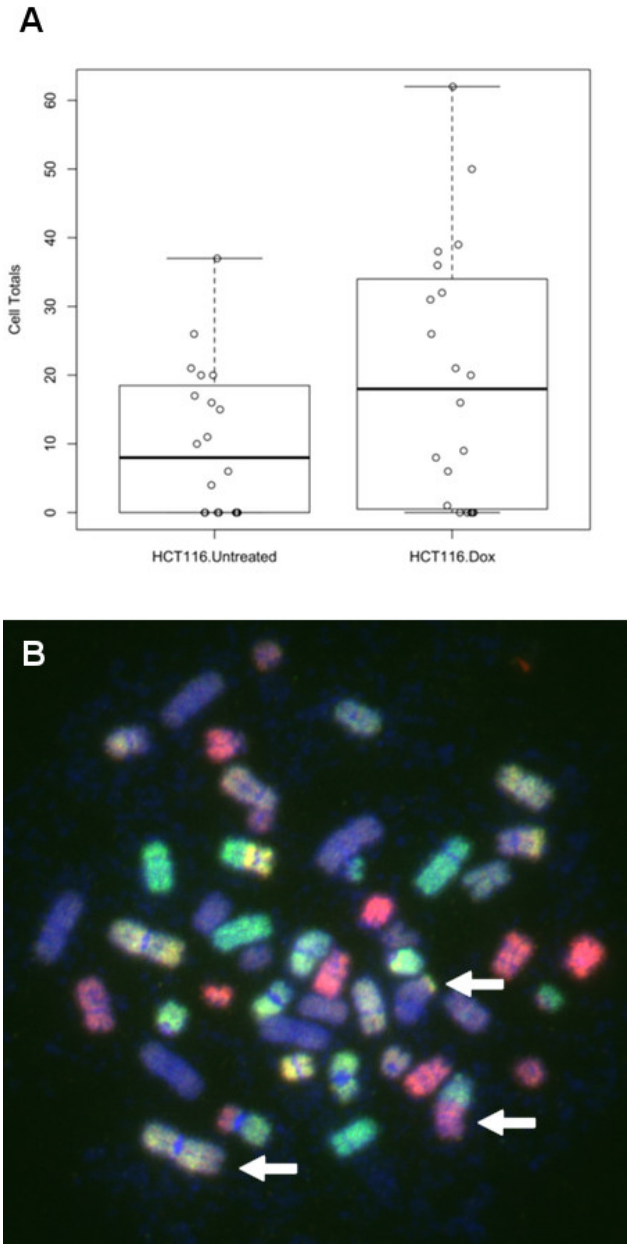


Figure 7: High-dose doxorubicin treatment results in increased single-cell derived population growth and karyotypic alteration. (A) In situ single cell growth of HCT116 cells post-doxorubicin treatment/recovery. Single cells (20 per sample) are identified and monitored daily for five days. Cell totals were calculated after five days of growth. (B) Multi-color chromosome painting karyotype image of recovered high-dose doxorubicin-treated HCT116 cell. Novel clonal translocations indicated by arrows. Although genome chaos is the mechanism that produces altered genomes, highly chaotic genomes are often not detectable in later stages.

pointing out that clones that finally emerge from this process often display much simpler alterations, possibly due to the fact that too many drastic genome changes result in cells that are either non-viable or viable. Despite that chaotic genomes are necessary for genome evolution, more stable genomes are selected from chaotic genome evolution. This combined approach (growth pattern analysis and karyotype analysis) demonstrates that these outliers do not exist within the cell population prior to treatment. It is important to point out that there are different strategies to profile emergent and original populations. One such approach is through DNA sequencing; however, another more effective way is through karyotyping, as the gene defines a feature of the system, while the karyotype defines the overall system itself. It is known that the karyotype codes for system inheritance while the gene codes for parts inheritance (Heng, Stevens et al. 2011).

Discussion

An identified, general macro-cellular evolutionary model of high-dose drug-induced cancer drug resistance

A number of conclusions can be reached from our analysis. These findings reveal a key transition in cancer drug resistance, which is summarized and illustrated with the following model (Figure 8): 1) high-dose therapeutics can sharply reduce tumor cell counts; however, these also induce increased genome chaos as a trade-off; 2) genome heterogeneity and evolutionary potential are increased through stochastic, rapid fragmentation and reorganization of chaotic genomes, followed by selection of stable, altered genomes. This treatment-

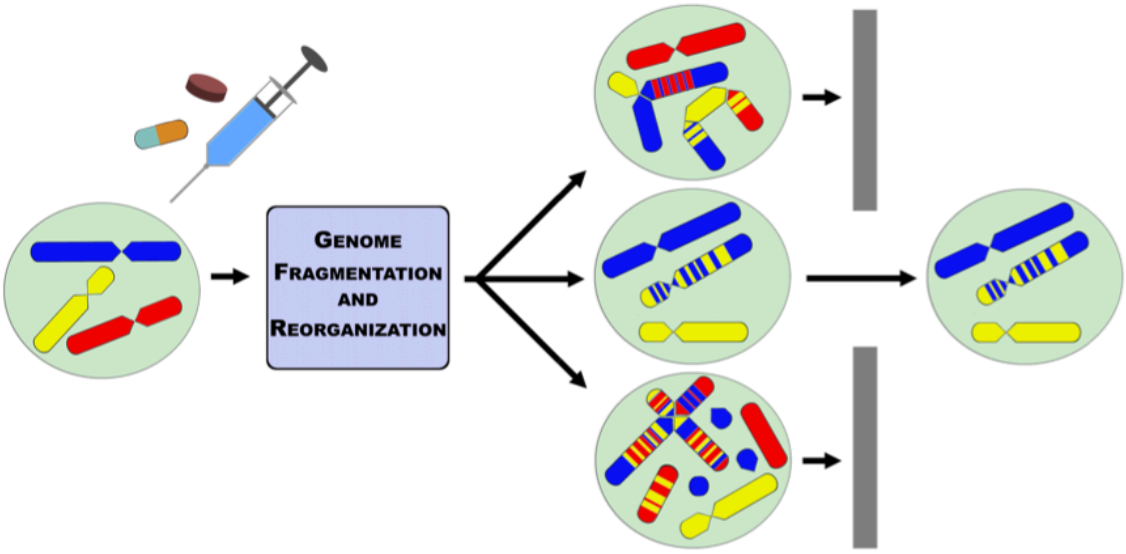


Figure 8: Diagram depicting the impact of high-dose treatment stress-induced chromosomal instability in the context of genome-mediated cancer evolution. Different chromosomes of the genome are designated by color (blue, yellow, red). Regardless of the targeted mechanism, high-dose drug-related stress to the genome results in rapid, stochastic genome fragmentation and genome topology reorganization. The consequences of this process are increased karyotypic heterogeneity and evolutionary potential, as shown by numerical aberrations (e.g. aneuploidy) and/or structural aberrations (e.g. translocations). Treatment-induced heterogeneity serves as a necessary precondition for selection. Stable karyotypes that survive this process are selected for clonal expansion, and aggressive subgroups will dominate tumor growth through rapid tumor cell repopulation.

induced increase of heterogeneity serves as the necessary pre-condition for the emergence of a small fraction of aggressive subgroups or outliers that dominate rapid tumor cell population growth after recovery; 3) these subgroups can then drive cancer progression through rapid generation of cancer cell populations, swiftly recovering lost tumor cell numbers initially eliminated by treatment (Abdallah, Horne et al. 2013).

To demonstrate that this model is also applicable to many other systems, a variety of cancers in vivo and in vitro were treated with various approaches, spanning different targeting strategies (Table 2). Pre-treatment and post-recovery tumor cells were analyzed by spectral karyotyping. In all cases, resistant clones displayed obvious genome change, demonstrating the emergence of new systems following drug treatment. We thus can conclude, without following all known therapeutic agents and approaches, that the end result of aggressive therapy will be similar; surviving cells will present altered genomes as the products of this general phenomenon. Interestingly, similar observations were recently reported in budding yeast, where the heterogeneity of aneuploidy cell populations increased with stress, causing resistance to emerge (Chen, Mulla et al. 2015). This macro-evolutionary model represents a general model of drug resistance in cancer, where induced genome chaos (not selection of pre-existing variants) is mainly responsible. It is now clear that induction of this drastic process is dependent on the degree of the treatment administered rather than the specific molecular mechanism that is targeted (Liu, Stevens et al.

2014), as specific targeting also leads to rapid drug resistance through this same genome-mediated mechanism. This may provide reasoning as to why long-term success stories from cancer specific therapeutic targeting are exceptional cases (Horne, Stevens et al. 2013).

Despite the low probability of generating outliers with aggressive growth (and presumably additional features), cancer prevails through strength in numbers (very high tumor cell counts in patients greatly increase the likelihood of this event occurring with each treatment administration). This represents an excellent example of how the law of truly large numbers combined with evolutionary selection power can always favor these unlikely events (Limbrick-Oldfield 2014). It is interesting to point out that, despite one-in-a-million events happening all the time, molecular biologists often choose to ignore infrequent events in their experimental design. The strategy of using averaging profiling and eliminating outliers (as these are commonly regarded as noise) has misled the field of cancer drug resistance. According to current standards of molecular characterization, 1 in 20 samples should be ignored. However, in clinical reality, a one-in-a-thousand event will likely be responsible for rapid drug resistance. Cancer research, therefore, must focus on the reality of large numbers of cancer cells, which make drug resistance a certainty despite the low probability of inducing successful outlier formation.

This transition encompasses the variety of known molecular mechanisms of drug resistance in cancer

Genome alteration reconciles the large number of diverse molecular mechanisms of drug resistance, most of these falling under the categories of genetic heterogeneity and pharmacokinetics (Heng 2015). Individual molecular mechanisms (e.g. specific mutations, pathway alterations, active multi-drug pumps) work for some subpopulations, such as those under physiological conditions, within micro-evolutionary phases, and especially in response to low-dose regimens, as reflected by average population behavior. However, when the tumor cell population is under crisis, such as during periods of aggressive therapy administration, only drastic genome reorganization can rapidly offer opportunity for survival. Thus, the game changes from fine-tuning existing systems by gene/epigene modifications to the formation of new survivable systems through genome reorganization, which explains why the vast majority of surviving tumor cells display altered genome-defined systems (Heng 2009; Heng, Stevens et al. 2011; Heng 2015; Stepanenko, Andreieva et al. 2015).

Using genome replacement to unify the molecular mechanisms of drug resistance also fits under the evolutionary mechanism of cancer (Heng, Stevens et al. 2010). Increased evidence supports this new framework. Recent glioma and ovarian cancer sequencing studies revealed different genetic landscapes, including karyotype-level change, of patient tumor cells prior to and after treatment (Johnson, Mazor et al. 2014; Patch, Christie et al. 2015). More importantly, massive genome alteration represents a major characteristic of the vast majority of cancer genomes following drug treatment, and genome

reorganization can overpower or change the context of gene mutations. Even though drug resistance in cancer is a dynamic process that occurs at multiple genetic levels, genome-mediated macro-cellular cancer evolution is the key (Heng, Stevens et al. 2006; Heng, Liu et al. 2010; Heng, Stevens et al. 2010; Heng, Stevens et al. 2011; Heng, Bremer et al. 2013). Initially observed by cytogenetic analyses, the concept of genome chaos during punctuated cancer evolution has been confirmed by large-scale cancer genome sequencing (Meyerson and Pellman 2011; Navin, Kendall et al. 2011; Stephens, Greenman et al. 2011; Holland and Cleveland 2012; Baca, Prandi et al. 2013; Wang, Waters et al. 2014).

Implications for cancer drug resistance research

Our results call upon new methodologies to study drug resistance based on this entire process (high initial cell death, genome reorganization, increased heterogeneity and outlier-driven progression) rather than initial treatment impact only. In addition, measuring genomic heterogeneity and stability status of the disease may provide pertinent information regarding potential treatment and appropriate treatment dosage. Current analytical methods neglect the contributions of outliers and overall heterogeneity to disease progression (Abdallah, Horne et al. 2013), so emphasis must be placed on applying single-cell resolution techniques to achieve a comprehensive understanding of disease stability and progression before and during treatment regimens. Further, this transition could be further understood with application of the multiple level

adaptive landscape model, as this directly illustrates the relationship between genome change (macro-evolution) and gene/epigene changes (micro-evolution) (Heng, Liu et al. 2011; Heng, Bremer et al. 2013; Huang 2013).

Implications in therapeutic regimen research, development and administration

This new understanding thus warrants strong consideration in the majority of therapeutic regimen research and development efforts. The clinical message from these studies is to avoid triggering genome chaos during intervention, because this phenomenon ultimately contributes to rapid recurrence and resistance. However, the current goal of improving clinical approaches is considered accomplished through the conception and application of drugs with increased initial response when compared to the standard line of care. Despite any improvement of initial efficacy, this study suggests that application of increasingly aggressive approaches will continue to trigger genome chaos and generate genomically and phenotypically different clones that will drive cancer progression.

Lowering initial numbers prior to treatment administration (i.e. through surgical removal of tumors) should safely limit induced outlier formation and resistance, as these studies have shown that this mechanism occurs at a low frequency. In ovarian cancer for example, studies show an association between survival and amount of postoperative residual disease (National Collaborating Centre for Cancer (UK) 2011). This new mechanism would suggest that successfully primary surgical removal of tumors, resulting in drastic reduction of

the tumor cell population, minimizes the odds of inducing aggressive outlier-driven recurrence when treatment is administered after surgery. Rather than developing more potent agents, efforts should continue to be placed on improving surgical techniques and early detection methods, as effective surgery clearly serves as the ideal measure for achieving long-term patient survival.

Finally, drug-induced genome chaos can provide explanation behind the early successes of alternative treatment strategies that do not incorporate the administration of maximum tolerated doses. These include adaptive therapy, where the goal is to maintain a stable tumor burden by adjusting treatments and dose concentrations in response to tumor cell growth/death (Gatenby, Silva et al. 2009; Gillies, Verduzco et al. 2012; Silva, Kam et al. 2012). Metronomic therapy aims to maximize tumor cell death, but this strategy involves a rhythmic regimen consisting of more frequent treatments than the maximum tolerated dose strategy, but of lower concentrations (Kerbel and Kamen 2004). These two approaches perhaps fulfill the new clinical directions we proposed, as both apply constraint to the tumor cell population with lower dose therapeutics and prolong overall survival. As our early results suggested (Heng et al. unpublished observations), lower concentrations of therapeutics may reduce both the onset of genome chaos and formation of aggressive outliers; however, this needs to be further explored. If this is the case, cancer maintenance through cancer genome constraint and overall tumor cell population management rather than maximizing

tumor cell death counts would offer greater long-term benefit and significantly extend patient life span.

CHAPTER 3: SEARCHING FOR A CONCEPTUAL FRAMEWORK TO STUDY THE PATTERN OF CANCER EVOLUTION THAT ACCOUNTS FOR HETEROGENEITY AND OUTLIER CONTRIBUTIONS

Introduction

In Chapter 2, a drug-induced transition was identified that illustrates a key clinical paradox between initial effective tumor cell killing with high-dose therapeutics and robust, long-term cancer drug resistance. This transition, representing a general mechanism of cancer drug resistance, comprised of 1) induced genome chaos (rapid genome fragmentation and reorganization); 2) increased genome heterogeneity, the necessary pre-condition for the formation of aggressive outliers; and 3) aggressive outlier-driven progression, represented by the emergence of new features such as rapid proliferation. This transition was identified through experimentation and analyses designed to monitor long-term system behavior and account for both heterogeneity (i.e. genome, cell growth) and outlier contributions rather than focusing on specific patterns and initial killing impact.

Cancer heterogeneity at multiple levels (e.g. genetic, metabolic, growth) is an often-recognized phenomenon in cancer research, yet it is frequently disregarded in studies in terms of experimental design, data collection/validation and in the synthesis of findings and conclusions. The strategy implemented by many researchers is to identify the signal within the noise using a wide variety of molecular techniques in order to identify key molecular mechanisms or pathways primarily responsible for cancer processes, key stages in progression and/or

features. This has been accomplished over the past few decades using average-based techniques to gather profiles of sampled tumor cell populations. Indeed, such strategy has been further supported by current statistical analyses that eliminate most outliers. Such approaches have also promoted the clonal evolutionary concept, which explains some exceptional cases such as the chronic phase of chronic myeloid leukemia (Horne, Stevens et al. 2013). However, clonal expansion-driven cancer evolution has been difficult to document in the majority of cancer cases.

By identifying the switching between the stepwise Darwinian phase (where somatic clonal expansion can be frequently observed) and stochastic punctuated cancer evolution (Heng, Stevens et al. 2006), this issue could have been solved. However, the majority of researchers were not convinced. Perhaps due to their excitement to find the long-expected pattern within the noise, they had hoped to identify the key genetic signature of cancer through the cancer genome sequencing project involving large numbers of clinical samples (Heng 2015). Disappointingly, the results of the current cancer genome sequencing project have only revealed increased heterogeneity at multiple genetic levels (Horne, Ye et al. 2015a; Heng 2015). Clearly, some basic concepts as they apply to cancer need to be re-investigated.

Recent work has demonstrated that the statistical mean may be ill-suited for profiling many pathological conditions, including cancer, with elevated genome heterogeneity (Abdallah, Horne et al. 2013). During the macro-cellular

evolutionary phase of cancer for example, the average cancer cell is non-existent, as system heterogeneity is dominant. Use of average-based analyses in this phase is incorrect, as averages eliminate diversity. This explains the data from the cancer genome sequencing project well, where many studies have failed to identify consistent drivers. In addition, when tracing cancer evolution, the patterns that are revealed are highly dynamic and punctuated, even at the DNA sequence level at single-cell resolution (Navin, Kendall et al. 2011; Wang, Waters et al. 2014; Sottoriva, Kang et al. 2015). As a result, a "big bang" model was recently introduced to account for the bursts of high intratumoral heterogeneity observed, and tumor progression has been described as "punctuated clonal expansions with few persistent intermediates" (Navin, Kendall et al. 2011; Sottoriva, Kang et al. 2015).

When cancer enters the micro-evolutionary phase; however, statistical averages may faithfully be employed, as system heterogeneity is lower, with the majority of change occurring at lower genetic levels (e.g. gene, epigene). This can explain why average-based approaches can accurately apply towards specific stages of linear models. This could also explain the punctuated phases and limited clonal expansions observed from sample to sample, as the two phases of cancer evolution progress in NCCA/CCA cycles (Heng, Stevens et al. 2006; Heng 2015).

Thus, the findings and approaches from the previous chapter raise questions regarding how to appropriately account for heterogeneity and outliers

in cancer research as well as determining which type(s) of statistical strategies should be applied, as knowing the practical value of these outliers is highly significant. More importantly, the approach we took in collecting and synthesizing data led to a crucial realization: a unique appreciation for outliers in cancer research is essential, and a new research framework/methodology is urgently needed to correctly study a system where heterogeneity is dominant. We thus utilize these valuable data to study scientific methodology in cancer.

Specifically, we comparatively analyzed the results of the same sets of experiments with two different frameworks of thinking to investigate the potentially significantly different conclusions. The first involves traditional thinking that eliminates outliers when their frequencies are low. The second is a holistic approach with the aim to monitor overall system behavior, where aggressive outliers represent essential drivers under high levels of macro-evolutionary selection despite their low likelihood of success. Through this theoretical synthesis, it is clear that application of different concepts and strategies leads to drastically different conclusions.

Different Analytical Approaches Lead to Drastically Different Conclusions

According to the strategy geared towards eliminating “noise,” when one out of three trials represents a positive result, such result should be ignored if it can be justified significantly, as it represents the minority. Similarly, the positive results from one out of twenty trials would surely be disregarded in analysis. However, as we presented in Chapter 2, the average treated cell totals post-

recovery were consistently lower than untreated controls in all six trials (Figure 4). This approach falls short in making the connection between initial cell death from aggressive treatment and robust, rapid long-term cancer drug resistance, as it cannot illustrate this transition in the first place. By disregarding the outliers in this study, we cannot justify the clinical reality that drug-induced resistant clones rapidly emerge, despite being successfully generated at extremely low frequencies. This represents a generally accepted approach in many molecular genetic studies. Statistical tools are utilized to analyze averaged patterns, and in doing so, the contributions of outliers to data sets are effectively reduced or eliminated.

In contrast, by accounting for and placing value on all data, even those at frequencies of 1/3 or 1/20, we were able to identify aggressive outliers and associate their contribution to overall tumor population growth and robust drug resistance. After performing three experiments observing the effects of long-term growth after different doses of doxorubicin, we observed faster cell growth in the high-dose group compared to untreated controls (Figure 3). In the other two experiments, we observed higher cell growth in the untreated control groups. As this experiment did not yield consistent results for these three trials, conventional thinking would dismiss these experiments without a conclusion. Further, these experiments fail to faithfully link to the clinical situation that robust drug resistance is clinical reality.

With the elimination of noise in analysis also comes the disregard for overall sample heterogeneity. In contrast, when we calculated coefficients of variation to illustrate overall cell total heterogeneity, we realized that the evolutionary potential of the treated samples was consistently elevated for all trials compared to untreated controls (Figure 4).

In contrast to identifying consistent explanations and average-based conclusions, we applied an evolutionary approach by focusing on evolutionary potential that reflects on both overall heterogeneity and outliers, despite their lower odds for success. In addition to consistently lower cell total averages of treated samples after treatment and recovery, we observed higher coefficients of variation across all trials. This was anticipated considering the stability of the untreated cell line we used in experimentation combined with the stochastic nature of drug-induced genome chaos, as this process generates different genome products each time. Taking stochasticity into account, we realized that while heterogeneity (i.e. genome and growth) was increased and induced by the administered treatment, it was only a precondition that could lead to robust drug resistance, especially considering the low numbers of cells we analyzed in culture compared to the many millions and billions of tumor cells that may be affecting patients upon treatment. We made this realization after observing the infrequent event of emergent aggressive outliers in two of the six trials (at an overall sample frequency of 1 in 20) that outgrew all corresponding control samples. Despite its low frequency, this event becomes a certainty when tumor

cell populations (such as those observed in metastatic cancer patients) are very large. Though this perspective, we were able to identify a general transition providing explanation for robust drug resistance and answer a key clinical paradoxical question that we were able to using average-based methods alone, as lower average cell totals could not extend to rapid, robust cancer drug resistance. This was accomplished with the detection of drug-induced, increased heterogeneity at multiple levels and the realization of the contribution of generated aggressive outliers to the overall tumor cell population post-recovery.

It is worth noting that disregarding heterogeneity and outliers in average-based analyses may reduce the need for increased sample/cell population sizes in experimental study, as associations/findings with statistical significance may be accomplished with a smaller sample size. If the frequencies of outliers are fixed, and all outliers would be eliminated by these approaches regardless, there would be no need to increase the sample size and number of trials. In Chapter 2, the majority of cells were initially eliminated from treatment in our study, and those that emerged would be ignored (and their contributions to overall tumor cell population progression after recovery would be underestimated) depending on the type of analysis (i.e. average-based analyses).

However, if emphasis is shifted towards capturing outliers, then expansion of sample sizes/trial numbers is essential, as expansion would reveal the overall range of data. In Chapter 2, this resulted in increased numbers of recovered

populations, increasing confidence of overall findings (consistently lower cell total average, yet emergent aggressive outliers).

In Chapter 2, we expanded experimentation to six total trials and the number of total samples to 60 to systematically observe this process. After treatment and recovery, we observed lower averages across treated samples in all trials compared with controls. Outlier treated samples were also detected that outgrew corresponding control groups, and this finding was key, as these identified outliers would be responsible for driving overall tumor population growth through increased rates of proliferation. At these low frequencies (1/20-1-60); however, these data could be ignored in the synthesis of conclusions, especially if the focus of the study is on the average-based findings. Average-based analysis would suggest that doxorubicin treatment is effective at slowing tumor cell growth after recovery compared to untreated tumor cell growth, and this could serve as part of the conclusion to the study. However, it is very important to note that this conclusion, where treatment is effective at slowing tumor cell population growth on average after recovery, does not provide explanation for the clinical problem of robust drug resistance, nor does it provide any direction or implication to solving this real clinical problem.

Outliers represent critical non-clonal chromosome aberrations and are the seeds for clonal chromosome aberrations later in cancer progression (Heng 2015). During genome chaos, each survivable NCCA continues to evolve. This chain of outliers, although undetectable by sequencing and averaging

heterogeneous populations, cannot be ignored. However, detection of outliers becomes obvious by examining karyotypic profiles at single-cell resolution, for example. Conventional thinking may suggest that outliers may be attributed to technical error; however, previous work has demonstrated that heterogeneity of a model system (e.g. growth rates of cell lines in continuous, long-term culture) or species can be inherited and maintained over generations (Abdallah, Horne et al. submitted). This recently introduced concept termed "fuzzy inheritance" suggests that these outliers are real, and given the roles these outliers play in a population in terms of progression and during times of crisis such as high-dose chemotherapeutic treatment and recovery, it is critical that these outliers be accounted for in cancer research analyses.

Defining key parameters for studying heterogeneous populations under stress

From this analysis, we have determined that there are three criteria that directly impact cell population heterogeneity-mediated drug resistance, and these should be considered when accounting for heterogeneity defined cancer evolutionary potential. The first is the internal stability of the cells within the population, which can be measured as single-cell resolution heterogeneity at various levels (e.g. karyotype, sequence, epigenetic). The second criterion is the environmental factors that can serve as stress to the population. We have previously discussed how environmental stress can induce increased population dynamics at multiple levels (Stevens, Abdallah et al. 2011; Stevens, Liu et al. 2014; Liu, Stevens et al. 2014; Horne, Chowdhury et al. 2014; Horne, Pollick et

al. 2015; Heng, Horne et al. 2016). Various stresses at high levels (e.g. chemotherapeutics, changes in culture conditions) have been associated with inducing chromosome fragmentation and genome chaos (Stevens, Liu et al. 2007; Stevens, Liu et al. 2014; Liu, Stevens et al. 2014). Finally, the third criterion is cell population size. As this study has shown, infrequent, drastic shifts in dynamics, such as high-dose treatment induced genome chaos and outlier-driven population growth, can be more readily observed in initially treated cells within larger population sizes.

Implications for Experimental Design and Data Presentation

This analysis offers implications for experimental design and data presentation. In order to appropriately capture any minority events that could impact population dynamics and long-term survival consequences, numbers of experimental trials should be increased. Considering current limited resources available (e.g. research funding, materials, patient samples, etc.), experimental efficiency has become important. This added pressure encourages performing the absolute minimum number of experimental trials necessary to achieve significant findings, which may be accomplished with just a few experimental trials. However, when dealing with highly dynamic, heterogeneous conditions and models, expansion of experimental trials and samples is necessary to determine the overall range of potential outcomes.

In the shift to using a holistic approach to monitor system behavior, all data points must be incorporated. This includes a needed shift away from using

methods that involve outlier removal in the pursuit of achieving statistical significance. In contrast, we cannot disregard the majority of data in order to fit a convenient story. This is the case for a landmark Rous sarcoma virus cell transformation study, where 80-90% of cells infected did not transform. However, the minority of cells that did transform was presented as the rule and not the exception. All data should be presented rather than selecting for exceptions and passing them as the general rule, even if this means that the overall message will become far more complicated.

In addition to experimental trial counts, trial durations must be extended to encapsulate entire biological processes. Using cancer drug treatment and resistance studies as an example, studies of potential new agents/therapeutic approaches are typically concluded after the initial impact (e.g. cell death counts or tumor shrinkage) is determined. For identifying molecular explanations of drug resistance, the window of observation is focused on tumor cells that have already undergone rounds of treatments and completed multiple NCCA/CCA cycles, and therapeutic resistance at the molecular level is determined based on end product analysis. However, information from both of these approaches (initial treatment impact and end product analysis) does not shed light on the overall process of the formation and emergence of resistant populations. Initial treatment impact does not directly translate to reduced mortality (Pepper 2011), and cancer drug resistance remains a real clinical problem despite continued efforts to increase maximum initial cell death counts. Specific molecular targeting approaches

derived from end product analyses also result in long-term drug resistance, so seeking out dominant pathways or highly expressed genes from resistant cells may not be the solution.

Research Focus Must be Shifted to Single Cell Level Resolution

We must also prioritize biological research down to the single cell level, and this prioritization has been emphasized recently with increased pressure from the National Institutes of Health with the introduction of the NIH Single Cell Analysis Program (https://www.nih.gov/news-events/news-releases/nih-common-fund-announces-awards-single-cell-analysis). Considering the vast heterogeneity observed by our group and others at the single cell level, we can no longer skew our understandings by averaging these population data. In cytogenetics, this extends beyond recording clonal chromosomal aberrations, but accounting for and analyzing non-clonal chromosomal aberrations. This will provide the total profile of the population in terms of dynamics and stability (Heng, Stevens et al. 2006; Heng, Regan et al. 2016; Heng, Horne et al. submitted).

Prioritization of Genetic Levels is Necessary for Constructing Accurate Understandings of Complex Diseases

In terms of dealing with the abundance of (and often conflicting) genetic information available, prioritization of genetic levels will be key to constructing understandings of diseases including cancer, and to remedy frustrations and unnecessary disagreements between groups that place focus on different levels. We have recently addressed this important question and proposed a strategy to

measure and account for molecular data at different genetic levels (Heng, Horne et al. submitted). In order to gain an overall system understanding at single cell resolution, we prioritized the genome level. Under genome theory, genome topology (sequence and three-dimensional architecture) defines the system inheritance or blueprint of an organism, whereas individual genes/epigenes only represent "parts inheritance," as these can be reorganized to form different systems (Heng, Stevens et al. 2010; Heng, Liu et al. 2011; Horne, Wexler et al. 2015; Horne, Ye et al. 2015a; Heng 2015). However, if the genome level within a population is stable (e.g. as evidenced by a homogeneous population characterized by clonal chromosomal aberrations), focus could then be shifted to lower genetic levels to gain further insight. However, as we have previously discussed, molecular universal understandings that are shared from case to case are extraordinarily rare, and will likely be identified in only the most homogeneous of disease conditions (e.g. Bcr-Abl fusion gene in chronic myeloid leukemia patients in the chronic phase) or physiological conditions (Horne, Stevens et al. 2013). It is also important to note that different pathways are involved in different phenotypes, as both redundancy and overlap exist, and this especially holds true in cancer (Stevens, Liu et al. 2014; Horne, Pollick et al. 2015). To summarize, the overall stability must be measured first to determine any potential utility of lower level genetic or average-based data.

Classification of Biomedical Research Studies Will Aide in Selecting Informative Analytical Approaches

In addition to prioritizing genetic levels, classification of biomedical research studies is necessary in order to determine an effective analytical strategy. For instance, average-based approaches provide data that are most accurate for determining patterns within cell populations of high homogeneity, as the signals that are easily identified are arguably representative of processes encompassing the cell population. However, with highly heterogeneous populations, where drastic variance may exist from cell-to-cell, genes/pathways identified within these populations may have conflicting roles, and averaging methods will provide misleading results. Further, outliers play key roles in heterogeneous populations, and these contributions will be underestimated or disregarded.

The categorization of biomedical research should effectively distinguish between systems/events of high and low heterogeneity to indicate to researchers whether average-based analytical approaches or accounting for heterogeneity and outlier contributions is the appropriate strategy. For example, average-based understandings apply well to physiological conditions, where genome constraint and homogeneity are high. This can also be extended to situations of low levels of stress, which may require an energy cost by cells to adapt or at most low levels of mutations (Horne, Chowdhury et al. 2014; Heng, Horne et al. 2016). In these conditions, mutations or minor aberrations are easily detected and representative of the patient's tissues/cells. In cancer, this strategy may also apply to the stepwise, micro-cellular evolutionary phase. Again, genome homogeneity is high in this phase, and any changes that occur during this phase

are traceable and readily identified by averaging methods (Heng, Stevens et al. 2006). These types of examples could be further examined and illustrated with application of the adaptive landscape concept, as these variations are minor, shared, and easily detected (Heng 2015).

In contrast, pathological conditions can be highly heterogeneous. The search for molecular causative mechanisms of common diseases has resulted in the detection of high levels of genome alterations (Horne, Chowdhury et al. 2014). Autism and Alzheimer's disease have been associated with altered karyotypes (Ye, Liu et al. 2007; Iourov, Vorsanova et al. 2008). Aneuploidy has been detected in several brain diseases (Iourov, Vorsanova et al. 2012a). Comparative genomic hybridization analysis has revealed that 80% of children with intellectual disability, autism, epilepsy and congenital abnormalities exhibited copy number variants, chromosomal imbalances or meiotic genome instability (Iourov, Vorsanova et al. 2012b). Stochastic genome alterations were observed in Gulf War Illness and chronic fatigue syndrome patients (Heng, Liu et al. 2013). Celiac and Crohn's disease patients display significantly increased numbers of chromosomal aberrations in peripheral blood lymphocytes (Hojsak, Gagro et al. 2013). Increased polyploidy has been observed in cardiomyocytes associated with hypertension, congenital heart disease and cardiac overloading (Davoli and de Lange 2011). These conditions differ from single gene or single network-driven disease, as highly penetrant genetic defects are not detectable within the patient population. For these conditions that display a high degree of diversity,

the heterogeneity and outliers should be taken into account under an evolutionary framework (Horne, Chowdhury et al. 2014).

This strategy also applies to the macro-cellular evolutionary phase of cancer, which is characterized by high levels of genome heterogeneity between tumor cells and progression is not traceable (Heng, Stevens et al. 2006). As Chapter 2 has demonstrated, accounting for heterogeneity and outliers is a useful approach for high stress conditions, such as aggressive chemotherapeutic treatment and recovery of tumor cells. These conditions of high heterogeneity, providing opportunity for outlier emergence and success, could be further examined and illustrated under the survival landscape concept (Heng 2015).

Why We Have Trouble Confirming Results Through Experiment Replication in Cancer Research (and Beyond)

This synthesis provides insight as to why researchers are having trouble repeating experiments and successfully confirming results in cancer research as well as other areas of biomedical and biological research. In cancer, average-based thinking does not apply to cancer dynamics where system reorganization and pathway switching are constantly involved. Recent work involving the reproduction of key 'landmark' experiments by scientists in the haematology and oncology department at the biotechnology firm Amgen has garnered a lot of attention throughout the scientific community, as out of a total of 53 experiments performed, the scientific findings were confirmed in only six (11%) (Begley and Ellis 2012). Rather than immediately placing blame on researchers' techniques or making any wild accusations, perhaps the heterogeneity of the models utilized

(both intra- and inter-model heterogeneity) and stochasticity could explain these failures to reproduce and achieve the original, precise findings. From what we have discussed, increasing the number of experimental trials carried out in each study could provide the true range of findings for these experiments, including the original data published and those obtained in the reproducibility study. In addition, the approaches we discussed above to properly account for heterogeneity should be applied, including observing and collecting data at a single cell resolution and accounting for all data points. The heterogeneity of the models should be determined as well, as it is clear that precise, predictable results are the products of only the most linear and stable models. Even HCT116 (the human colon cancer cell line studied in Chapter 2) displays a low degree of heterogeneity despite documented as a very karyotypically stable cancer cell line (Thompson, Compton et al. 2008; Knutsen, Padilla-Nash et al. 2010; Abdallah, Horne et al. 2013). Through model profiling, experimental trial/sample size expansion, and accounting for variance and outliers in heterogeneous models/samples, researchers should have more success in validating their findings, as experimentation with heterogeneous models results in ranges of outcomes.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

Application of genome theory resulted in the identification of a general, drug-induced macro-cellular evolutionary mechanism of cancer drug resistance

In this project, we identified a key transition in cancer drug resistance that bridges the current clinical paradoxical gap between initially effective drug-induced cell death and rapid, robust drug resistance. This transition was summarized with the following model: 1) clinically-relevant, high-dose therapeutics can sharply reduce tumor cell counts (initially); however, as a trade-off, this high stress also induces increased genome chaos (reflecting a genome-mediated survival strategy beyond lower-level adaptation); 2) both overall genome heterogeneity and aggressive outlier numbers are increased through rapid, stochastic fragmentation and formation of chaotic genomes, followed by the selection of stable, altered genomes. This treatment-induced increase of heterogeneity serves as the necessary pre-condition for the emergence of a small fraction of aggressive subgroups or outliers that dominate rapid tumor cell population growth after recovery; 3) these subgroups can then drive cancer progression through rapid generation of cancer cell populations, swiftly recovering lost tumor cell numbers initially eliminated by treatment (Abdallah, Horne et al. 2013). The novelty of these findings is the demonstration of the impact of drug-induced karyotypic alteration (or macro-cellular evolution) in cancer drug resistance, which is the formation and selection of new, outlier genetic systems that effectively drive overall tumor cell population growth post-

treatment and recovery, as evidenced by observed drug-induced karyotypic alteration and rapid proliferation. This represents a general mechanism of cancer drug resistance, as genome level alteration has been observed in patients, in vitro and in vivo models and the result of various therapeutic approaches (Heng, Stevens et al. 2006; Stevens, Liu et al. 2007; Ye, Liu et al. 2007; Stephens, Greenman et al. 2011; Stevens, Abdallah et al. 2011; Baca, Prandi et al. 2013). In fact, this result has been predicted by the genome theory of cancer evolution, which focuses on high stress-mediated macro-cellular evolution (Heng 2015).

Much focus in the field of cancer research has been placed on identifying key gene or epigene markers of cancer drug resistance, and genome-level alterations have previously been disregarded as artifacts (Heng 2015). However, the presence of chaotic genomes has been recently confirmed in many cancers (Heng, Liu et al. 2011; Heng, Stevens et al. 2011; Stephens, Greenman et al. 2011; Baca, Prandi et al. 2013). This project thus provides evidence that cancer drug resistance is the direct consequence of drug-induced genetic dynamics at multiple levels, and alterations at the genome level play a crucial role. According to the evolutionary mechanism of cancer, which is equal to the collection of all diverse molecular mechanisms (Ye, Stevens et al. 2009; Heng, Stevens et al. 2010; Heng, Stevens et al. 2011; Heng, Bremer et al. 2013), each individual molecular mechanism can be unified at the genome level, as the genome represents a macro-evolutionary selection unit. Even though an individual

molecular mechanism can effectively promote cellular adaptation, it is insufficient for the cell to survive under extremely high stress conditions. The cell needs genome change (i.e. new system formation) to save the day.

Establishment of a conceptual framework to study the pattern of cancer evolution that accounts for heterogeneity and outlier contributions

In Chapter 3, we raised questions regarding how to appropriately account for heterogeneity and outliers in cancer research in light of the role these have in cancer drug resistance. This was rather important, as we were able to show that application of different analytical approaches (i.e. average-based analyses vs. heterogeneity and outlier-focused analysis) led to drastically different approaches. Importantly, the clinical paradoxical gap between high initial cell death with high-dose therapy and rapid, robust drug resistance was bridged after growth heterogeneity and aggressive outliers were accounted for.

We defined key criteria for analyzing heterogeneous populations in studies. These were the following: 1) the internal stability of the cells within a population, which can be accomplished through measuring heterogeneity of multiple genetic levels at single-cell resolution; 2) environmental factors that provide stress to the population, as these influence system dynamics; and 3) cell population size, as infrequent, drastic shifts in dynamics can be more readily observed in large population sizes (e.g. high-dose treatment induced genome chaos and outlier-driven population growth).

We extended this synthesis to biomedical studies and offered additional approaches to aide in both selecting informative analytical approaches and

designing experiments when the issue of heterogeneity is prevalent. These included the expansion of trial numbers and sample sizes to capture any minority (yet potentially highly influential) events, shifting research focus down to single-cell level resolution, prioritizing genetic levels and classifying biomedical research studies. This synthesis also offered explanation behind the failure to confirm some results through experiment replication, as experimentation with heterogeneous models can result in a range of outcomes.

Implications of drug-induced genome-mediated cancer drug resistance on current large-scale research efforts, treatment administration and cancer drug development

Current large-scale efforts including the Moon Shots Program launched in 2012 have a simple, yet ambitious, aim to "end the threat of cancer" (www.cancermoonshots.org). This bold move will be carried out through the acquisition and analysis of massive data sets combined with the development and customization of panels of therapeutic agents. Utilization of impressive state-of-the-art technologies, strong collaborations and abundant resources aside, the overall mission of these types of programs has not changed since the beginning of cancer treatment administration decades ago: cancer must be eliminated.

However, as we have shown, high levels of stress such as those associated with aggressive therapeutic regimens induce genome chaos-mediated cancer evolution, including general or specific-targeting treatment agents (Stevens, Abdallah et al. 2011; Liu, Stevens et al. 2014). While the

magnitude of these projects and the sophistication of the technologies utilized are certainly appealing, application of the same goals and framework from the past few decades may yield similar results. For example, while high-throughput sequencing can identify candidate molecular targets, and precisely designed drugs can effectively eliminate massive numbers of cancer cells initially, genome chaos can create new survivable systems with completely different genetic profiles, making the initially administered targeted therapy now off-target. To explain this situation, we have recently applied cancer evolutionary theory to unify the hallmarks of cancer (Figure 9) (Horne, Pollick et al. 2015).

Based on the conclusions from this project and previous efforts, the degree of stress (e.g. drug concentration) introduced to cancer cells plays the major role in triggering genome chaos as opposed to the specific marker that is directly targeted (Stevens, Abdallah et al. 2011; Liu, Stevens et al. 2014). This is critical information for both drug development and treatment administration, as current emphasis is on the application of precision medicine and administration of combination therapy in order to maximize tumor cell death counts. Regardless of the therapeutic approach selected, these studies would suggest that aggressive therapy in many forms would result in the same outcome: genome chaos induction and outlier-driven robust drug resistance.

Understanding that administration of the maximum tolerated dose is the current standard of care for most patients, considerations should be made with regards to alternative therapeutic regimens to avoid treatment-induced, genome

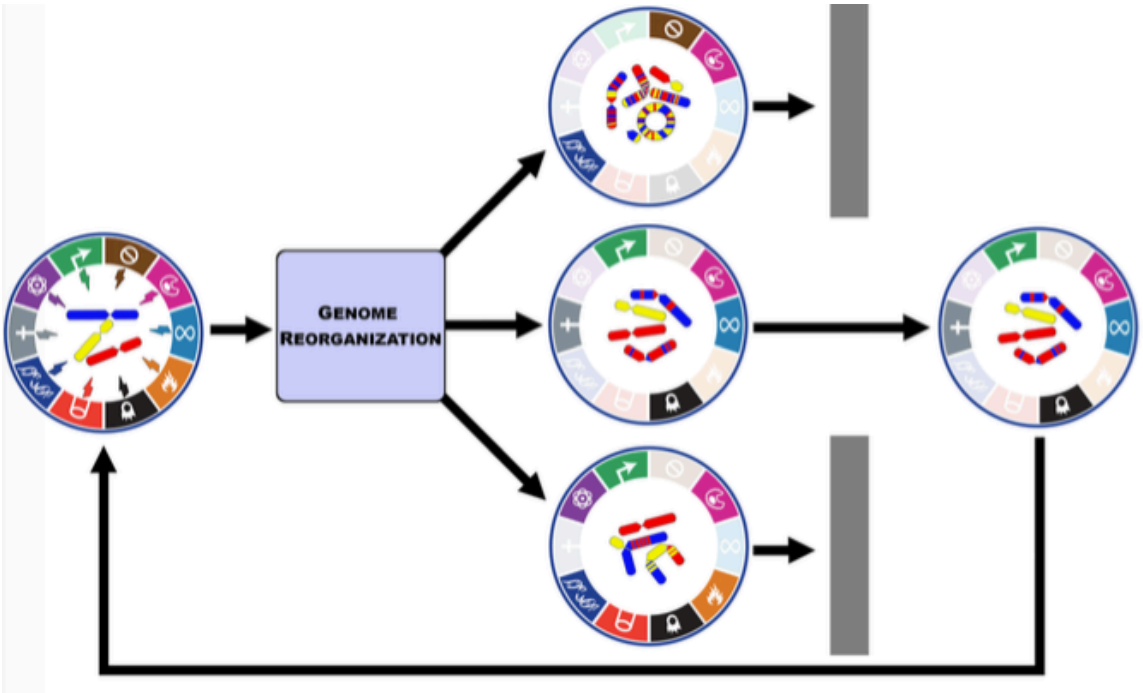


Figure 9. Diagram depicting the impact of stress-induced chromosomal instability in the context of genome-mediated cancer evolution. Different chromosomes of the genome are designated by color (red, blue, yellow). The hallmarks of cancer (shown surrounding the chromosomes, hallmark symbols derived from Hanahan and Weinberg, 2011) can serve as sources of stress to the genome as represented by lightning bolts, resulting in rapid, stochastic genome fragmentation and genome topology reorganization. The consequences of this process are increased karyotypic heterogeneity and evolutionary potential, as shown by numerical aberrations (e.g. aneuploidy) and/or structural aberrations (e.g. translocations). Breakdown of system constraints and alteration of genome topology result in phenotypic variance, as represented by different highlighted hallmark combinations for each genome system. Stable karyotypes that survive this process are selected for clonal expansion, and this process cycles upon future internal/external crisis events. Reproduced from Horne, Pollick et al. 2015 with permission from John Wiley & Sons Inc.

chaos-driven cancer drug resistance in patients. As previously discussed, regimens including adaptive therapy and metronomic therapy, which both involve the administration of lower dosages than the maximum tolerated dose, have shown early success (Kerbel and Kamen 2004; Gatenby, Silva et al. 2009; Silva, Kam et al. 2012). We propose that perhaps these successes could be explained by the findings of this study and of future efforts, where lower doses of therapeutics could effectively constrain tumor cell counts without inducing genome chaos-mediated macro-cellular evolution. Lower dose regimens could still potentially induce alterations at other levels (e.g. gene mutations, epigenetic alterations), representing cancer drug resistance at a micro-cellular evolutionary level. We have previously proposed the relationship of different degrees of generalized stress and their impact on different genetic levels (Figure 10) (Liu, Stevens et al. 2014; Horne, Chowdhury et al. 2014; Horne, Pollick et al. 2015; Heng 2015; Heng, Horne et al. 2016). The impact of low-dose therapeutics on lower levels of genetic dynamics must be further explored as well as the determination of thresholds that induce genome chaos in order to maintain genome constraint while safely and efficiently eliminating tumor cells. Finally, patient tumor samples should be analyzed prior to treatment in an effort to profile the overall cancer genome stability of a patient. We have observed higher instances of induced genome chaos after treating cells displaying higher genome instability (Liu, Stevens et al. 2014). This critical measurement could contribute to the development of a more beneficial treatment plan to patients displaying

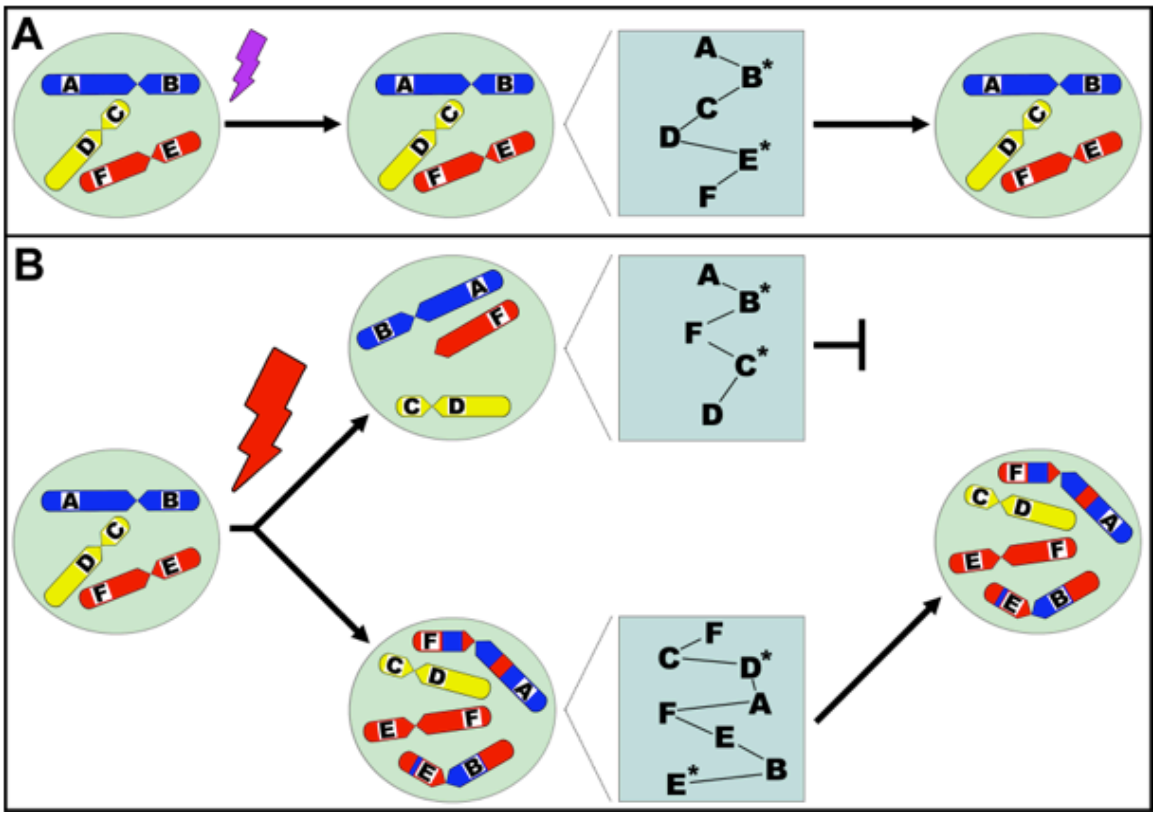


Figure 10. Diagram illustrating the relationship between stress, genome topology alteration, resulting genetic network reorganization, and successful evolutionary selection. Different chromosomes are designated by color (red, yellow, blue) and drawn within the nucleus, representing the genome, and genes are designated A, B, C, D, E, F within the chromosomes. Corresponding protein networks are illustrated by the relationships between proteins A, B, C, D, E, F. A cell is exposed to a moderate level of stress (A), resulting in genetic and/or epigenetic alteration as indicated by asterisks (*) next to impacted proteins. The cell survives the stress event without genome-level alteration. When a cell is exposed to a high level of stress (B), this results in genome topology alteration represented by numerical aberrations (e.g. aneuploidy) and/or structural aberrations (e.g. translocations). This directly affects the physical three-dimensional relationship between genes and changes the overall genetic network structure, resulting in drastic systemic changes beyond the influence of genetic and/or epigenetic alterations that may concurrently occur. As a consequence, the corresponding protein network changes are shown by altered relationships between proteins. These new genomic systems then undergo evolutionary selection, and those that are stochastically selected upon may clonally expand and dominate the cell population. Reproduced from Horne, Chowdhury et al. 2014 with permission from Frontiers Media S.A.

unstable cancer genomes while avoiding the unnecessary (and potentially detrimental) administration of harsh treatment.

Expansion of this project by incorporation additional models and treatment strategies will confirm the general role of drug-induced macro-cellular evolution in cancer drug resistance

In this project, we utilized an in vitro model designed to watch evolution-in-action in order to identify the transition between effective initial cell killing with high-dose doxorubicin and rapid drug resistance. To validate our findings and confirm this outlier-driven transition as a general phenomenon, this project should be expanded to include the administration of high doses of various therapeutic agents. Expansion of this study would also incorporate the testing and monitoring of additional cell lines and in vivo models. Ideally, patient tumor samples would be collected and analyzed pre- and post-treatment in an attempt to capture this transition in the clinic.

Identification of a treatment "balancing act" consisting of cancer genome constraint and tumor cell population control would provide insight for future treatment regimen development

Another future direction for this study is the confirmation of cancer genome level constraint with lower doses of therapeutic agents (i.e. lower cell killing without the induction of genome chaos), providing explanation for the earlier successes of alternative regimens that involve lower dose treatments. This could be accomplished by designing future experiments involving a panel of agents at various concentrations to search for a treatment balance, where certain degrees of drug-related stress are capable of controlling tumor cell population

counts, but these are not too high to trigger genome chaos-mediated cancer drug resistance.

Extensive profiling of aggressive outliers and genome chaos will be necessary to further understand the relationship between different levels of genetic change and emergent features

As described in Chapter 2, the identification of drug-induced aggressive outliers was accomplished through cell growth monitoring of single-cell derived populations after treatment and recovery. We also demonstrated that aggressive outliers displayed altered karyotypes as the result of drug-related stress-induced genome chaos. We further demonstrated that altered genomes display elevated transcriptome dynamics (Stevens, Horne et al. 2013; Stevens, Liu et al. 2014; Horne, Liu et al. submitted). However, many questions remained unanswered regarding the profiles of these outlier groups. This includes the identification of gene mutations and impacted pathways with single-cell level DNA sequencing and determining the contributions of these in terms of increased genome level re-organization and cancer drug resistance. In addition to rapid proliferation, additional features including metabolic profiles, immune evasion and migration could be assessed and linked to the findings from genetic profiling. This type of analysis could reveal key general mechanistic understandings underlying the phenomena we observed at the karyotype level.

Specifically, single-cell resolution sequencing technologies could be employed to trace the entire process of drug-induced genome chaos, for example. This would include sequencing genomes of tumor cells during key

stages (e.g. tumor genomes prior to treatment, chaotic genomes present shortly after treatment, and altered, stable genomes after re-organization and selection) followed by the identification of genes and pathways that could be linked to karyotypic alteration (e.g. stress pathways, DNA repair mechanisms, gene mutation patterns that are essential for genome re-organization). Furthermore, based on our current in vitro analyses with limited chemotherapeutics, parallel experiments must be examined to include more specific molecular targeting agents to demonstrate the same mechanisms. Such understanding is crucial for the field to accept this reality of cancer drug resistance, as it is not just associated with chemotherapeutics, but rather all interventions that aim to kill cancer cells. Alternative approaches that aim to manage cancer (rather than maximize cell death) need to be examined. Examples include changing the evolutionary pattern or speed to constrain cancer cells and paying more attention to tissue and higher systems above the individual cell level. Moreover, animal models and patient samples must be systematically examined based on our in vitro findings. One potential clinical strategy is to profile general genome instability for patients to predict the likelihood of genome chaos, and careful consideration must be made when considering maximum tolerated dose regimens for subsets of patients that display unstable cancer genomes. These proposed directions should identify some order within the chaos, improve our odds in the “War on Cancer,” and deepen our understanding of cancer drug resistance.

APPENDIX A: HEMACYTOMETER-BASED CELL COUNTING RESULTS OF SIX DOXORUBICIN HIGH-DOSE TREATMENT TRIALS

Trial 1	C1	C2	C3	C4	C1-4 Sum	C5	C6	C7	C8	C5-8 Sum	Sum Avg	D.F.	Total
Control 1.1	41	32	32	32	137	29	34	46	58	167	152	0.5	1.9x10^5
Control 1.2	23	25	32	51	131	42	37	35	33	147	139	0.5	1.7x10^5
Control 1.3	31	35	36	48	150	21	33	32	25	111	130.5	0.5	1.6x10^5
Control 1.4	28	26	41	27	122	27	20	42	23	112	117	0.5	1.5x10^5
Control 2.1	39	28	36	37	140	23	31	61	46	161	150.5	0.5	1.9x10^5
Control 2.2	13	32	50	42	137	35	36	32	26	129	133	0.5	1.7x10^5
Control 2.3	43	27	45	30	145	37	36	35	20	128	136.5	0.5	1.7x10^5
Control 2.4	35	18	26	40	119	35	60	24	49	168	143.5	0.5	1.8x10^5
Control 3.1	30	29	39	50	148	56	51	27	36	170	159	0.5	2.0x10^5
Control 3.2	41	28	52	59	180	58	33	52	32	175	177.5	0.5	2.2x10^5
Control 3.3	30	28	43	41	142	58	52	29	36	175	158.5	0.5	2.0x10^5
Control 3.4	44	29	59	43	175	60	54	37	32	183	179	0.5	2.2x10^5
Control 4.1	26	29	58	39	152	40	36	58	32	166	159	0.5	2.0x10^5
Control 4.2	32	34	24	45	135	31	26	26	22	105	120	0.5	1.5x10^5
Control 4.3	40	29	28	70	167	42	62	41	33	178	172.5	0.5	2.2x10^5
Control 4.4	20	40	38	47	145	29	41	35	34	139	142	0.5	1.8x10^5
Sample 1.1	30	25	25	22	102	29	50	23	38	140	121	0.5	1.5x10^5
Sample 1.2	16	18	23	18	75	29	27	20	11	87	81	0.5	1.0x10^5
Sample 1.3	25	10	22	19	76	22	13	14	19	68	72	0.5	9.0x10^4
Sample 1.4	15	3	12	12	42	21	11	10	24	66	54	0.25	3.4x10^4
Sample 2.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 2.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 2.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 2.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 3.1	74	56	100	116	346	91	96	53	63	303	324.5	0.5	4.1x10^5
Sample 3.2	57	57	89	68	271	89	89	69	60	307	289	0.5	3.6x10^5
Sample 3.3	62	67	103	102	334	92	66	81	64	303	318.5	0.5	4.0x10^5
Sample 3.4	48	23	14	41	126	78	67	53	36	234	180	0.5	2.3x10^5
Sample 4.1	0	0	0	0	0	0	0	0	0	0	0	0	0

Sample 4.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 4.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 4.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 5.1	31	24	27	35	117	45	34	38	38	155	136	0.5	1.7x10^5
Sample 5.2	46	49	36	45	176	51	55	48	49	203	189.5	0.5	2.4x10^5
Sample 5.3	26	29	31	26	112	18	39	48	29	134	123	0.5	1.5x10^5
Sample 5.4	55	64	101	73	293	102	91	57	69	319	306	0.5	3.8x10^5
Sample 6.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 6.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 6.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 6.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 7.1	15	13	17	20	65	9	14	17	11	51	58	0.5	7.3x10^4
Sample 7.2	14	15	17	14	60	21	24	15	13	73	66.5	0.5	8.3x10^4
Sample 7.3	17	9	9	12	47	25	23	14	22	84	65.5	0.5	8.2x10^4
Sample 7.4	12	9	16	11	48	9	21	12	18	60	54	0.5	6.8x10^4
Sample 8.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 8.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 8.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 8.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 9.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 9.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 9.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 9.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 10.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 10.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 10.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 10.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Trial 2	**C1**	**C2**	**C3**	**C4**	**C1-4 Sum**	**C5**	**C6**	**C7**	**C8**	**C5-8 Sum**	**Sum Avg**	**D.F.**	**Total**
Control 5.1	6	12	12	10	40	12	13	4	11	40	40	0.5	5.0x10^4
Control 5.2	18	19	26	34	97	37	37	13	24	111	104	0.5	1.3x10^5
Control 5.3	14	11	14	5	44	14	19	20	13	66	55	0.5	6.9x10^4
Control 5.4	12	6	12	11	41	17	10	11	6	44	42.5	0.5	5.3x10^4

Control 6.1	66	58	83	87	294	88	112	74	75	349	321.5	0.5	4.0x10^5
Control 6.2	32	33	30	39	134	36	44	41	30	151	142.5	0.5	1.8x10^5
Control 6.3	40	42	76	72	230	82	98	48	64	292	261	0.5	3.3x10^5
Control 6.4	47	33	75	64	219	53	57	37	36	183	201	0.5	2.5x10^5
Control 7.1	59	73	72	120	324	112	124	64	59	359	341.5	0.5	4.3x10^5
Control 7.2	72	57	64	92	285	48	58	39	37	182	233.5	0.5	2.9x10^5
Control 7.3	57	63	129	121	370	114	102	77	42	335	352.5	0.5	4.4x10^5
Control 7.4	38	35	52	70	195	68	36	48	50	202	198.5	0.5	2.5x10^5
Control 8.1	51	49	104	104	308	90	104	57	44	295	301.5	0.5	3.8x10^5
Control 8.2	41	41	74	64	220	89	46	53	64	252	236	0.5	3.0x10^5
Control 8.3	63	81	92	110	346	105	91	58	63	317	331.5	0.5	4.1x10^5
Control 8.4	56	47	79	78	260	74	72	68	42	256	258	0.5	3.2x10^5
Sample 11.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 11.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 11.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 11.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 12.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 12.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 12.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 12.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 13.1	23	21	33	30	107	27	32	19	19	97	102	0.5	1.3x10^5
Sample 13.2	20	12	32	25	89	25	27	18	25	95	92	0.5	1.2x10^5
Sample 13.3	16	13	33	33	95	23	23	10	17	73	84	0.5	1.1x10^5
Sample 13.4	9	12	13	10	44	11	12	13	8	44	44	0.5	5.5x10^4
Sample 14.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 14.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 14.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 14.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 15.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 15.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 15.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 15.4	0	0	0	0	0	0	0	0	0	0	0	0	0

Sample 16.1	16	14	29	34	93	23	25	25	15	88	90.5	0.5	1.1x10^5
Sample 16.2	8	12	17	20	57	12	15	13	12	52	54.5	0.5	6.8x10^4
Sample 16.3	14	22	23	20	79	28	25	13	20	86	82.5	0.5	1.0x10^5
Sample 16.4	16	19	21	33	89	26	17	17	11	71	80	0.5	1.0x10^5
Sample 17.1	18	14	22	26	80	39	33	25	18	115	97.5	0.25	6.1x10^4
Sample 17.2	18	20	28	37	103	21	35	18	20	94	98.5	0.25	6.2x10^4
Sample 17.3	21	18	30	16	85	29	20	29	25	103	94	0.25	5.9x10^4
Sample 17.4	30	15	26	30	101	33	32	16	24	105	103	0.25	6.4x10^4
Sample 18.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 18.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 18.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 18.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 19.1	11	14	18	11	54	9	19	9	19	56	55	0.25	3.4x10^4
Sample 19.2	6	13	20	17	56	20	33	18	10	81	68.5	0.25	4.3x10^4
Sample 19.3	12	21	18	13	64	25	20	5	15	65	64.5	0.25	4.0x10^4
Sample 19.4	11	14	19	37	81	28	22	25	29	104	92.5	0.25	5.8x10^4
Sample 20.1	11	21	18	8	58	13	15	15	12	55	56.5	0.25	3.5x10^4
Sample 20.2	10	7	10	8	35	10	5	9	8	32	33.5	0.25	2.1x10^4
Sample 20.3	3	15	8	1	27	10	5	9	8	32	29.5	0.25	1.8x10^4
Sample 20.4	16	2	3	3	24	12	7	13	4	36	30	0.25	1.9x10^4
Trial 3	**C1**	**C2**	**C3**	**C4**	**C1-4 Sum**	**C5**	**C6**	**C7**	**C8**	**C5-8 Sum**	**Sum Avg**	**D.F.**	**Total**
Control 9.1	41	38	42	41	162	40	75	42	36	193	177.5	0.5	2.2x10^5
Control 9.2	54	56	123	93	326	43	63	41	55	202	264	0.5	3.3x10^5
Control 9.3	50	29	58	65	202	42	56	45	33	176	189	0.5	2.4x10^5
Control 9.4	26	39	58	46	169	32	45	38	44	159	164	0.5	2.1x10^5
Control 10.1	49	39	37	40	165	30	26	24	27	107	136	0.5	1.7x10^5
Control 10.2	51	29	45	62	187	33	32	18	25	108	147.5	0.5	1.8x10^5
Control 10.3	35	42	43	38	158	35	29	50	29	143	150.5	0.5	1.9x10^5
Control 10.4	38	44	40	35	157	34	25	34	40	133	145	0.5	1.8x10^5
Control 11.1	25	42	44	28	139	40	16	24	22	102	120.5	0.5	1.5x10^5
Control 11.2	41	34	48	31	154	23	29	35	32	119	136.5	0.5	1.7x10^5
Control 11.3	21	22	24	21	88	32	34	29	21	116	102	0.5	1.3x10^5

Control 11.4	24	23	45	29	121	26	19	14	15	74	97.5	0.5	1.2x10^5
Control 12.1	43	25	22	29	119	48	38	49	29	164	141.5	0.5	1.8x10^5
Control 12.2	27	33	46	59	165	53	46	29	40	168	166.5	0.5	2.1x10^5
Control 12.3	22	32	39	33	126	39	52	34	41	166	146	0.5	1.8x10^5
Control 12.4	44	46	30	20	140	30	34	24	32	120	130	0.5	1.6x10^5
Sample 21.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 21.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 21.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 21.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 22.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 22.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 22.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 22.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 23.1	22	27	16	21	86	17	16	15	25	73	79.5	0.25	5.0x10^4
Sample 23.2	23	25	23	21	92	29	24	29	27	109	100.5	0.25	6.3x10^4
Sample 23.3	28	9	24	19	80	23	15	25	45	108	94	0.25	5.9x10^4
Sample 23.4	16	26	20	28	90	10	36	34	30	110	100	0.25	6.3x10^4
Sample 24.1	20	15	21	27	83	26	17	29	24	96	89.5	0.25	5.6x10^4
Sample 24.2	13	8	20	15	56	22	13	31	19	85	70.5	0.25	4.4x10^4
Sample 24.3	21	17	12	17	67	21	16	8	8	53	60	0.25	3.8x10^4
Sample 24.4	24	28	9	19	80	19	24	18	15	76	78	0.25	4.9x10^4
Sample 25.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 25.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 25.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 25.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 26.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 26.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 26.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 26.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 27.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 27.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 27.3	0	0	0	0	0	0	0	0	0	0	0	0	0

Sample 27.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 28.1	56	52	90	65	263	72	63	47	36	218	240.5	0.5	3.0x10^5
Sample 28.2	61	74	105	102	342	87	88	58	53	286	314	0.5	3.9x10^5
Sample 28.3	41	88	73	86	288	72	60	109	84	325	306.5	0.5	3.8x10^5
Sample 28.4	47	47	87	67	248	77	81	58	59	275	261.5	0.5	3.3x10^5
Sample 29.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 29.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 29.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 29.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 30.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 30.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 30.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 30.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Trial 4	**C1**	**C2**	**C3**	**C4**	**C1-4 Sum**	**C5**	**C6**	**C7**	**C8**	**C5-8 Sum**	**Sum Avg**	**D.F.**	**Total**
Control 13.1	45	49	63	70	227	72	71	34	43	220	223.5	0.5	2.8x10^5
Control 13.2	42	56	85	59	242	67	78	39	40	224	233	0.5	2.9x10^5
Control 13.3	33	27	55	59	174	57	62	37	41	197	185.5	0.5	2.3x10^5
Control 13.4	45	30	23	33	131	38	37	30	26	131	131	0.5	1.6x10^5
Control 14.1	39	24	51	37	151	51	50	31	44	176	163.5	0.5	2.0x10^5
Control 14.2	30	29	63	58	180	34	41	31	33	139	159.5	0.5	2.0x10^5
Control 14.3	21	11	23	23	78	40	42	32	25	139	108.5	0.5	1.4x10^5
Control 14.4	21	32	29	20	102	20	35	27	25	107	104.5	0.5	1.3x10^5
Control 15.1	38	39	50	51	178	36	44	50	40	170	174	0.25	1.1x10^5
Control 15.2	53	84	93	81	311	103	97	99	79	378	344.5	0.25	2.2x10^5
Control 15.3	67	95	109	122	393	120	126	71	72	389	391	0.25	2.4x10^5
Control 15.4	88	78	117	143	426	110	119	68	104	401	413.5	0.25	2.6x10^5
Control 16.1	31	44	57	52	184	55	60	56	43	214	199	0.5	2.5x10^5
Control 16.2	60	46	63	53	222	79	64	42	54	239	230.5	0.5	2.9x10^5
Control 16.3	45	41	70	62	218	63	95	49	52	259	238.5	0.5	3.0x10^5
Control 16.4	32	34	51	44	161	47	41	36	34	158	159.5	0.5	2.0x10^5
Sample 31.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 31.2	0	0	0	0	0	0	0	0	0	0	0	0	0

Sample 31.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 31.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 32.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 32.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 32.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 32.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 33.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 33.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 33.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 33.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 34.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 34.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 34.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 34.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 35.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 35.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 35.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 35.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 36.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 36.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 36.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 36.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 37.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 37.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 37.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 37.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 38.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 38.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 38.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 38.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 39.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 39.2	0	0	0	0	0	0	0	0	0	0	0	0	0

Sample 39.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 39.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 40.1	25	22	26	33	106	38	51	28	37	154	130	0.5	1.6x10^5
Sample 40.2	40	37	26	25	128	38	37	25	24	124	126	0.5	1.6x10^5
Sample 40.3	25	29	19	36	109	42	63	38	22	165	137	0.5	1.7x10^5
Sample 40.4	36	32	73	53	194	62	55	34	43	194	194	0.5	2.4x10^5
Trial 5	**C1**	**C2**	**C3**	**C4**	**C1-4 Sum**	**C5**	**C6**	**C7**	**C8**	**C5-8 Sum**	**Sum Avg**	**D.F.**	**Total**
Control 17.1	105	79	146	133	463	122	125	59	93	399	431	0.5	5.4x10^5
Control 17.2	74	80	128	110	392	127	109	92	69	397	394.5	0.5	4.9x10^5
Control 17.3	49	57	45	47	198	97	83	92	64	336	267	0.5	3.3x10^5
Control 17.4	55	54	89	98	296	95	88	106	93	382	339	0.5	4.2x10^5
Control 18.1	67	37	86	59	249	105	85	70	80	340	294.5	0.5	3.7x10^5
Control 18.2	51	40	46	46	183	74	71	70	63	278	230.5	0.5	2.9x10^5
Control 18.3	78	62	61	55	256	93	77	59	78	307	281.5	0.5	3.5x10^5
Control 18.4	75	84	59	53	271	85	90	96	90	361	316	0.5	4.0x10^5
Control 19.1	84	106	135	142	467	129	115	89	87	420	443.5	0.5	5.5x10^5
Control 19.2	46	43	79	75	243	90	93	61	54	298	270.5	1	6.8x10^5
Control 19.3	42	56	93	82	273	93	119	48	58	318	295.5	1	7.4x10^5
Control 19.4	58	46	110	102	316	125	101	47	57	330	323	1	8.1x10^5
Control 20.1	49	41	79	99	268	99	83	42	59	283	275.5	1	6.9x10^5
Control 20.2	36	39	45	38	158	66	62	45	34	207	182.5	1	4.6x10^5
Control 20.3	49	42	47	49	187	82	70	54	54	260	223.5	1	5.6x10^5
Control 20.4	33	38	56	42	169	58	66	34	42	200	184.5	1	4.6x10^5
Sample 41.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 41.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 41.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 41.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 42.1	9	17	12	20	58	17	13	14	9	53	55.5	0.5	7.0x10^4
Sample 42.2	12	9	12	13	46	13	12	9	14	48	47	0.5	5.9x10^4
Sample 42.3	17	13	17	22	69	33	21	15	21	90	79.5	0.5	9.9x10^4
Sample 42.4	15	13	32	19	79	22	18	17	12	69	74	0.5	9.3x10^4
Sample 43.1	0	0	0	0	0	0	0	0	0	0	0	0	0

Sample 43.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 43.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 43.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 44.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 44.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 44.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 44.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 45.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 45.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 45.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 45.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 46.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 46.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 46.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 46.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 47.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 47.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 47.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 47.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 48.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 48.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 48.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 48.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 49.1	29	29	32	22	112	31	36	40	45	152	132	0.5	1.7x10^5
Sample 49.2	31	23	39	37	130	44	41	30	43	158	144	0.5	1.8x10^5
Sample 49.3	51	41	42	26	160	62	47	42	45	196	178	0.5	2.2x10^5
Sample 49.4	17	20	18	29	84	35	45	31	44	155	119.5	0.5	1.5x10^5
Sample 50.1	56	57	102	92	307	83	95	68	54	300	303.5	0.5	3.8x10^5
Sample 50.2	71	45	117	128	361	116	115	69	69	369	365	0.5	4.6x10^5
Sample 50.3	78	93	119	113	403	170	136	78	83	467	435	0.5	5.4x10^5
Sample 50.4	74	96	155	155	480	82	112	82	88	364	422	0.5	5.3x10^5
Trial 6	**C1**	**C2**	**C3**	**C4**	**C1-4 Sum**	**C5**	**C6**	**C7**	**C8**	**C5-8 Sum**	**Sum Avg**	**D.F.**	**Total**

Control 21.1	43	51	51	55	200	88	100	59	60	307	253.5	0.5	3.2x10^5
Control 21.2	62	49	102	91	304	114	87	56	61	318	311	0.5	3.9x10^5
Control 21.3	40	40	80	80	240	75	71	46	58	250	245	0.5	3.1x10^5
Control 21.4	59	63	85	56	263	95	88	76	61	320	291.5	0.5	3.6x10^5
Control 22.1	79	63	58	74	274	93	77	55	55	280	277	0.5	3.5x10^5
Control 22.2	53	44	64	45	206	47	66	48	40	201	203.5	0.5	2.5x10^5
Control 22.3	61	62	100	82	305	75	89	74	68	306	305.5	0.5	3.8x10^5
Control 22.4	67	78	121	101	367	120	129	83	77	409	388	0.5	4.9x10^5
Control 23.1	72	52	59	52	235	71	78	43	45	237	236	0.5	3.0x10^5
Control 23.2	30	39	42	41	152	41	66	36	43	186	169	0.5	2.1x10^5
Control 23.3	31	39	37	30	137	41	48	36	40	165	151	0.5	1.9x10^5
Control 23.4	36	37	31	38	142	63	66	58	56	243	192.5	0.5	2.4x10^5
Control 24.1	34	43	39	41	157	60	61	57	36	214	185.5	0.5	2.3x10^5
Control 24.2	35	46	74	70	225	59	69	27	27	182	203.5	0.5	2.5x10^5
Control 24.3	54	57	73	87	271	126	98	60	51	335	303	0.5	3.8x10^5
Control 24.4	58	70	98	89	315	102	84	73	60	319	317	0.5	4.0x10^5
Sample 51.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 51.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 51.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 51.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 52.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 52.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 52.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 52.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 53.1	29	27	49	49	154	54	63	45	29	191	172.5	0.5	2.2x10^5
Sample 53.2	18	24	22	36	100	29	40	32	21	122	111	0.5	1.4x10^5
Sample 53.3	28	16	25	28	97	30	41	35	29	135	116	0.5	1.5x10^5
Sample 53.4	23	11	36	34	104	25	40	17	31	113	108.5	0.5	1.4x10^5
Sample 54.1	21	16	22	30	89	28	36	24	18	106	97.5	0.5	1.2x10^5
Sample 54.2	21	18	24	24	87	30	21	10	11	72	79.5	0.5	9.9x10^4
Sample 54.3	25	18	30	14	87	25	21	19	19	84	85.5	0.5	1.1x10^5
Sample 54.4	22	15	18	23	78	15	24	23	27	89	83.5	0.5	1.0x10^5

Sample 55.1	17	11	22	11	61	18	22	23	16	79	70	0.5	8.8x10^4
Sample 55.2	17	12	14	21	64	14	17	13	14	58	61	0.5	7.6x10^4
Sample 55.3	8	14	15	16	53	15	16	11	12	54	53.5	0.5	6.7x10^4
Sample 55.4	10	11	11	14	46	21	15	8	18	62	54	0.5	6.8x10^4
Sample 56.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 56.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 56.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 56.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 57.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 57.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 57.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 57.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 58.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 58.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 58.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 58.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 59.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 59.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 59.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 59.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 60.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 60.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 60.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Sample 60.4	0	0	0	0	0	0	0	0	0	0	0	0	0

C1-C8: Count 1 – Count 8; D.F.: Dilution factor; Treated samples that did not recover during the recovery period were given zero values.

APPENDIX B: HEMACYTOMETER-BASED CELL COUNTING RESULTS OF THREE DOXORUBICIN LOW-DOSE TREATMENT TRIALS

Trial 1	C1	C2	C3	C4	C1-4 Sum	C5	C6	C7	C8	C5-8 Sum	Sum Avg	D.F.	Total
Control 1.1	53	54	86	99	292	47	48	67	50	212	252	0.5	3.2x10^5
Control 1.2	67	54	75	83	279	80	114	62	61	317	298	0.5	3.7x10^5
Control 1.3	58	58	109	99	324	80	69	68	64	281	302.5	0.5	3.8x10^5
Control 1.4	54	50	66	61	231	79	76	67	60	282	256.5	0.5	3.2x10^5
Control 2.1	46	43	57	50	196	83	50	48	43	224	210	0.5	2.6x10^5
Control 2.2	50	54	71	67	242	46	49	36	52	183	212.5	0.5	2.7x10^5
Control 2.3	34	51	41	42	168	64	59	36	40	199	183.5	0.5	2.3x10^5
Control 2.4	71	43	83	74	271	54	72	58	42	226	248.5	0.5	3.1x10^5
Control 3.1	61	67	78	83	289	85	91	49	67	292	290.5	0.5	3.6x10^5
Control 3.2	65	67	90	119	341	115	94	63	60	332	336.5	0.5	4.2x10^5
Control 3.3	57	64	74	88	283	70	65	72	73	280	281.5	0.5	3.5x10^5
Control 3.4	60	55	61	88	264	56	64	74	74	268	266	0.5	3.3x10^5
Sample 1.1	44	61	46	74	225	47	55	54	59	215	220	0.5	2.8x10^5
Sample 1.2	62	43	98	76	279	79	35	34	47	195	237	0.5	3.0x10^5
Sample 1.3	41	43	59	66	209	77	73	62	31	243	226	0.5	2.8x10^5
Sample 1.4	40	32	96	64	232	51	33	38	47	169	200.5	0.5	2.5x10^5
Sample 2.1	55	47	83	85	270	67	68	59	63	257	263.5	0.5	3.3x10^5
Sample 2.2	66	68	102	101	337	80	79	75	62	296	316.5	0.5	4.0x10^5
Sample 2.3	59	66	80	129	334	101	141	65	58	365	349.5	0.5	4.4x10^5
Sample 2.4	45	43	61	73	222	81	109	55	55	300	261	0.5	3.3x10^5
Sample 3.1	84	68	107	123	382	124	112	85	67	388	385	0.5	4.8x10^5
Sample 3.2	88	82	68	113	351	77	97	73	69	316	333.5	0.5	4.2x10^5
Sample 3.3	87	76	113	97	373	161	157	82	109	509	441	0.5	5.5x10^5
Sample 3.4	77	75	96	144	392	84	95	85	93	357	374.5	0.5	4.7x10^5
Sample 4.1	73	57	96	65	291	56	66	44	60	226	258.5	0.5	3.2x10^5
Sample 4.2	54	62	88	94	298	51	66	68	39	224	261	0.5	3.3x10^5
Sample 4.3	83	70	101	91	345	129	108	71	86	394	369.5	0.5	4.6x10^5
Sample 4.4	88	70	95	133	386	74	155	63	75	367	376.5	0.5	4.7x10^5
Sample 5.1	23	19	27	27	96	17	44	22	33	116	106	0.5	1.3x10^5

Sample 5.2	28	32	48	48	156	44	72	46	50	212	184	0.5	2.3x10^5
Sample 5.3	40	27	38	38	143	52	41	28	51	172	157.5	0.5	2.0x10^5
Sample 5.4	37	30	47	28	142	36	41	26	28	131	136.5	0.5	1.7x10^5
Sample 6.1	39	46	43	44	172	55	50	43	40	188	180	0.5	2.3x10^5
Sample 6.2	51	52	51	50	204	49	49	51	51	200	202	0.5	2.5x10^5
Sample 6.3	51	48	49	50	198	51	64	54	54	223	210.5	0.5	2.6x10^5
Sample 6.4	69	63	68	56	256	61	54	56	51	222	239	0.5	3.0x10^5
Sample 7.1	38	34	50	40	162	35	28	45	37	145	153.5	0.5	1.9x10^5
Sample 7.2	31	29	59	53	172	64	69	37	45	215	193.5	0.5	2.4x10^5
Sample 7.3	62	60	57	63	242	86	88	48	50	272	257	0.5	3.2x10^5
Sample 7.4	51	48	60	68	227	46	37	47	48	178	202.5	0.5	2.5x10^5
Sample 8.1	58	40	54	39	191	43	46	39	56	184	187.5	0.5	2.3x10^5
Sample 8.2	44	41	59	78	222	45	47	45	46	183	202.5	0.5	2.5x10^5
Sample 8.3	58	67	53	37	215	83	85	48	65	281	248	0.5	3.1x10^5
Sample 8.4	61	57	70	64	252	49	54	33	59	195	223.5	0.5	2.8x10^5
Sample 9.1	69	38	35	52	194	91	60	64	60	275	234.5	0.5	2.9x10^5
Sample 9.2	59	55	78	72	264	57	91	68	58	274	269	0.5	3.4x10^5
Sample 9.3	53	56	51	37	197	84	88	52	71	295	246	0.5	3.1x10^5
Sample 9.4	56	42	57	69	224	41	46	50	60	197	210.5	0.5	2.6x10^5
Sample 10.1	94	86	117	95	392	101	101	76	89	367	379.5	0.5	4.7x10^5
Sample 10.2	111	103	129	119	462	102	156	99	79	436	449	0.5	5.6x10^5
Sample 10.3	98	59	119	111	387	72	75	67	88	302	344.5	0.5	4.3x10^5
Sample 10.4	89	52	91	77	309	119	132	81	83	415	362	0.5	4.5x10^5
Trial 2	**C1**	**C2**	**C3**	**C4**	**C1-4 Sum**	**C5**	**C6**	**C7**	**C8**	**C5-8 Sum**	**Sum Avg**	**D.F.**	**Total**
Control 4.1	43	29	39	38	149	74	63	38	50	225	187	0.5	2.3x10^5
Control 4.2	44	57	45	35	181	73	59	39	49	220	200.5	0.5	2.5x10^5
Control 4.3	50	47	41	44	182	107	71	61	51	290	236	0.5	3.0x10^5
Control 4.4	34	32	69	51	186	68	57	37	31	193	189.5	0.5	2.4x10^5
Control 5.1	60	46	31	30	167	74	79	42	47	242	204.5	0.5	2.6x10^5
Control 5.2	60	62	66	59	247	60	57	45	50	212	229.5	0.5	2.9x10^5
Control 5.3	60	60	63	75	258	41	59	42	47	189	223.5	0.5	2.8x10^5
Control 5.4	43	54	48	49	194	77	58	39	43	217	205.5	0.5	2.6x10^5

Control 6.1	42	51	73	51	217	54	46	39	32	171	194	0.5	2.4x10^5
Control 6.2	26	46	42	56	170	65	57	63	46	231	200.5	0.5	2.5x10^5
Control 6.3	51	41	85	76	253	62	72	34	54	222	237.5	0.5	3.0x10^5
Control 6.4	45	51	52	58	206	82	73	41	62	258	232	0.5	2.9x10^5
Control 7.1	40	42	57	71	210	75	69	39	42	225	217.5	0.5	2.7x10^5
Control 7.2	40	37	43	43	163	55	54	41	37	187	175	0.5	2.2x10^5
Control 7.3	37	34	75	68	214	41	47	52	50	190	202	0.5	2.5x10^5
Control 7.4	41	39	52	47	179	66	80	45	37	228	203.5	0.5	2.5x10^5
Sample 11.1	17	23	26	33	99	43	37	26	26	132	115.5	0.5	1.4x10^5
Sample 11.2	23	18	24	19	84	23	35	14	32	104	94	0.5	1.2x10^5
Sample 11.3	23	25	27	38	113	32	28	25	23	108	110.5	0.5	1.4x10^5
Sample 11.4	17	16	22	18	73	17	13	11	17	58	65.5	0.5	8.2x10^4
Sample 12.1	54	46	72	81	253	46	43	54	56	199	226	0.5	2.8x10^5
Sample 12.2	50	62	46	50	208	63	93	61	46	263	235.5	0.5	2.9x10^5
Sample 12.3	37	42	65	74	218	75	78	41	53	247	232.5	0.5	2.9x10^5
Sample 12.4	36	31	56	53	176	42	43	37	33	155	165.5	0.5	2.1x10^5
Sample 13.1	62	37	71	58	228	85	77	43	43	248	238	0.5	3.0x10^5
Sample 13.2	46	49	56	47	198	73	86	49	54	262	230	0.5	2.9x10^5
Sample 13.3	55	44	82	77	258	82	69	63	48	262	260	0.5	3.3x10^5
Sample 13.4	70	56	51	52	229	93	73	56	64	286	257.5	0.5	3.2x10^5
Sample 14.1	40	41	71	55	207	66	51	46	41	204	205.5	0.5	2.6x10^5
Sample 14.2	35	42	36	46	159	52	55	48	37	192	175.5	0.5	2.2x10^5
Sample 14.3	44	49	42	36	171	55	63	34	46	198	184.5	0.5	2.3x10^5
Sample 14.4	37	34	54	50	175	45	42	43	24	154	164.5	0.5	2.1x10^5
Sample 15.1	46	50	60	52	208	55	50	43	40	188	198	0.5	2.5x10^5
Sample 15.2	38	43	72	50	203	70	60	45	46	221	212	0.5	2.7x10^5
Sample 15.3	51	50	88	94	283	55	50	44	39	188	235.5	0.5	2.9x10^5
Sample 15.4	45	36	54	52	187	71	42	55	63	231	209	0.5	2.6x10^5
Sample 16.1	38	41	62	58	199	54	63	39	34	190	194.5	0.5	2.4x10^5
Sample 16.2	24	54	43	44	165	64	56	76	60	256	210.5	0.5	2.6x10^5
Sample 16.3	35	31	56	63	185	59	64	33	43	199	192	0.5	2.4x10^5
Sample 16.4	42	50	54	48	194	48	36	44	44	172	183	0.5	2.3x10^5

Sample 17.1	61	52	92	75	280	102	79	30	44	255	267.5	0.5	3.3x10^5
Sample 17.2	57	59	54	57	227	82	97	43	47	269	248	0.5	3.1x10^5
Sample 17.3	39	30	53	51	173	55	48	40	38	181	177	0.5	2.2x10^5
Sample 17.4	44	28	75	73	220	94	64	53	45	256	238	0.5	3.0x10^5
Sample 18.1	48	49	60	64	221	86	102	59	39	286	253.5	0.5	3.2x10^5
Sample 18.2	36	42	61	62	201	41	37	46	41	165	183	0.5	2.3x10^5
Sample 18.3	41	26	56	50	173	75	85	48	53	261	217	0.5	2.7x10^5
Sample 18.4	48	59	41	45	193	46	35	48	52	181	187	0.5	2.3x10^5
Sample 19.1	52	37	45	45	179	59	61	37	33	190	184.5	0.5	2.3x10^5
Sample 19.2	38	35	62	52	187	52	53	32	42	179	183	0.5	2.3x10^5
Sample 19.3	59	48	50	53	210	79	60	49	31	219	214.5	0.5	2.7x10^5
Sample 19.4	41	43	76	57	217	39	54	39	41	173	195	0.5	2.4x10^5
Sample 20.1	50	71	112	86	319	56	68	53	54	231	275	0.5	3.4x10^5
Sample 20.2	53	47	85	83	268	81	94	68	38	281	274.5	0.5	3.4x10^5
Sample 20.3	82	46	74	76	278	94	94	72	57	317	297.5	0.5	3.7x10^5
Sample 20.4	59	58	86	90	293	84	92	64	81	321	307	0.5	3.8x10^5
Trial 3	**C1**	**C2**	**C3**	**C4**	**C1-4 Sum**	**C5**	**C6**	**C7**	**C8**	**C5-8 Sum**	**Sum Avg**	**D.F.**	**Total**
Control 8.1	21	16	15	21	73	17	36	22	22	97	85	0.5	1.1x10^5
Control 8.2	23	22	41	43	129	28	26	19	24	97	113	0.5	1.4x10^5
Control 8.3	15	17	18	16	66	14	17	15	15	61	63.5	0.5	7.9x10^4
Control 8.4	16	23	28	22	89	14	21	15	7	57	73	0.5	9.1x10^4
Control 9.1	33	58	67	51	209	73	73	48	66	260	234.5	0.5	2.9x10^5
Control 9.2	69	56	86	93	304	69	81	50	55	255	279.5	0.5	3.5x10^5
Control 9.3	58	64	85	58	265	87	77	59	40	263	264	0.5	3.3x10^5
Control 9.4	62	61	70	57	250	77	80	63	68	288	269	0.5	3.4x10^5
Control 10.1	50	48	77	54	229	97	103	55	48	303	266	0.5	3.3x10^5
Control 10.2	61	48	61	63	233	106	91	63	53	313	273	0.5	3.4x10^5
Control 10.3	49	55	84	77	265	97	100	91	71	359	312	0.5	3.9x10^5
Control 10.4	33	55	63	69	220	50	56	64	47	217	218.5	0.5	2.7x10^5
Control 11.1	40	49	68	79	236	66	90	34	38	228	232	0.5	2.9x10^5
Control 11.2	46	37	44	54	181	58	55	36	29	178	179.5	0.5	2.2x10^5
Control 11.3	47	39	50	34	170	50	49	47	42	188	179	0.5	2.2x10^5

Control 11.4	28	31	51	50	160	27	32	44	40	143	151.5	0.5	1.9x10^5
Sample 21.1	48	46	70	43	207	78	67	43	31	219	213	0.5	2.7x10^5
Sample 21.2	52	46	58	73	229	57	67	49	38	211	220	0.5	2.8x10^5
Sample 21.3	44	56	77	87	264	44	49	42	48	183	223.5	0.5	2.8x10^5
Sample 21.4	37	32	32	33	134	28	36	32	30	126	130	0.5	1.6x10^5
Sample 22.1	31	26	47	37	141	51	27	29	27	134	137.5	0.5	1.7x10^5
Sample 22.2	17	37	65	65	184	45	29	25	31	130	157	0.5	2.0x10^5
Sample 22.3	46	46	77	73	242	54	41	46	46	187	214.5	0.5	2.7x10^5
Sample 22.4	59	61	81	72	273	48	53	40	39	180	226.5	0.5	2.8x10^5
Sample 23.1	38	32	65	73	208	29	34	35	33	131	169.5	0.5	2.1x10^5
Sample 23.2	25	17	19	22	83	33	24	17	25	99	91	0.5	1.1x10^5
Sample 23.3	39	22	34	35	130	28	26	35	38	127	128.5	0.5	1.6x10^5
Sample 23.4	33	31	50	24	138	55	40	41	37	173	155.5	0.5	1.9x10^5
Sample 24.1	36	30	33	37	136	34	40	37	29	140	138	0.5	1.7x10^5
Sample 24.2	38	65	81	84	268	69	91	48	41	249	258.5	0.5	3.2x10^5
Sample 24.3	35	35	42	45	157	93	76	54	57	280	218.5	0.5	2.7x10^5
Sample 24.4	43	38	40	48	169	43	39	36	37	155	162	0.5	2.0x10^5
Sample 25.1	34	36	44	50	164	50	47	47	45	189	176.5	0.5	2.2x10^5
Sample 25.2	32	36	46	35	149	52	50	32	33	167	158	0.5	2.0x10^5
Sample 25.3	40	32	40	43	155	31	38	30	39	138	146.5	0.5	1.8x10^5
Sample 25.4	23	38	28	31	120	36	54	29	25	144	132	0.5	1.7x10^5
Sample 26.1	25	29	49	48	151	49	38	30	29	146	148.5	0.5	1.9x10^5
Sample 26.2	28	35	35	38	136	58	52	24	23	157	146.5	0.5	1.8x10^5
Sample 26.3	36	34	46	51	167	63	71	36	56	226	196.5	0.5	2.5x10^5
Sample 26.4	32	33	32	38	135	30	45	35	32	142	138.5	0.5	1.7x10^5
Sample 27.1	54	49	83	80	266	79	85	56	55	275	270.5	0.5	3.4x10^5
Sample 27.2	43	41	62	72	218	66	71	44	40	221	219.5	0.5	2.7x10^5
Sample 27.3	57	50	91	88	286	95	84	46	77	302	294	0.5	3.7x10^5
Sample 27.4	53	47	35	55	190	60	55	38	61	214	202	0.5	2.5x10^5
Sample 28.1	32	30	49	38	149	49	45	40	48	182	165.5	0.5	2.1x10^5
Sample 28.2	28	30	21	26	105	33	34	36	30	133	119	0.5	1.5x10^5
Sample 28.3	35	25	46	49	155	46	39	35	31	151	153	0.5	1.9x10^5

Sample 28.4	37	41	37	41	156	56	50	34	32	172	164	0.5	2.1x10^5
Sample 29.1	30	51	39	36	156	34	40	24	34	132	144	0.5	1.8x10^5
Sample 29.2	18	30	29	40	117	46	49	22	21	138	127.5	0.5	1.6x10^5
Sample 29.3	32	28	49	59	168	83	52	21	31	187	177.5	0.5	2.2x10^5
Sample 29.4	21	22	18	17	78	22	17	15	22	76	77	0.5	9.6x10^4
Sample 30.1	43	55	62	50	210	54	47	40	58	199	204.5	0.5	2.6x10^5
Sample 30.2	44	63	80	59	246	48	57	63	57	225	235.5	0.5	2.9x10^5
Sample 30.3	36	50	89	96	271	96	71	42	48	257	264	0.5	3.3x10^5
Sample 30.4	47	55	92	111	305	115	110	62	64	351	328	0.5	4.1x10^5

C1-C8: Count 1 – Count 8; D.F.: Dilution factor.

APPENDIX C: SINGLE CELL-DERIVED POPULATION GROWTH OF UNTREATED AND RECOVERED HCT116 CELLS

HCT116.Untreated	Day 1	Day 2	Day 3	Day 4	Day 5
Sample 1	1	2	5	5	6
Sample 2	1	2	4	17	20
Sample 3	1	2	0	0	0
Sample 4	1	0	0	0	0
Sample 5	1	2	3	3	4
Sample 6	1	2	4	11	17
Sample 7	1	2	1	2	0
Sample 8	1	0	0	0	0
Sample 9	1	4	6	7	11
Sample 10	1	1	4	8	16
Sample 11	1	2	5	10	20
Sample 12	1	0	0	0	0
Sample 13	1	2	4	8	10
Sample 14	1	2	5	14	21
Sample 15	1	2	3	0	0
Sample 16	1	1	1	0	0
Sample 17	1	2	4	13	26
Sample 18	1	2	4	9	15
Sample 19	1	2	8	17	37
Sample 20	1	2	2	1	0
HCT116.Dox	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Sample 1	1	3	4	7	9
Sample 2	1	2	3	5	6
Sample 3	1	4	8	26	62
Sample 4	1	2	8	15	20
Sample 5	1	0	0	0	0
Sample 6	1	4	8	22	36
Sample 7	1	2	7	18	32
Sample 8	1	2	4	7	8
Sample 9	1	2	2	0	0
Sample 10	1	0	0	0	0
Sample 11	1	2	8	16	31
Sample 12	1	1	3	8	16
Sample 13	1	0	0	0	0
Sample 14	1	1	0	0	0

Sample 15	1	2	9	14	21
Sample 16	1	2	2	1	1
Sample 17	1	3	7	13	26
Sample 18	1	3	8	25	50
Sample 19	1	2	8	20	39
Sample 20	1	2	8	20	38

APPENDIX D: HORNE SD, STEVENS JB, ET AL. (2013). "WHY IMATINIB REMAINS AN EXCEPTION OF CANCER RESEARCH." J CELL PHYSIOL 228(4): 665-670

MINI-REVIEW

Journal of Cellular Physiology

Why Imatinib Remains an Exception of Cancer Research

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The archetype driving the drug targeting approach to cancer therapy is the success of imatinib against chronic phase chronic myeloid leukemia (CML-CP). Molecular targeting success of this magnitude has yet to be repeated for most solid tumors. To answer why imatinib remains an exception of cancer research, we summarize key features and patterns of evolution that contrast CML-CP from prostate cancer, an example of a solid tumor that also shares a signature fusion gene. Distinctive properties of CML-CP include: a large cell population size that is not geographically constrained, a highly penetrant dominant oncogene that sweeps the entire cell population, subsequent progressive and ordered clonal genetic changes, and the effectiveness of molecular targeting within the chronic phase, which is comparable to the benign phase of solid tumors. CML-CP progression resembles a clonal, stepwise model of evolution, whereas the pattern of solid tumor evolution is highly dynamic and stochastic. The distinguishing features and evolutionary pattern of CML-CP support why the success of imatinib does not carry over to most solid tumors. Changing the focus of cancer research from a gene-based view to a genome-based theory will provide insight into solid tumor evolutionary dynamics.

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Chronic myeloid leukemia (CML) is a hematological disorder characterized by uncontrolled proliferation of cells of the myeloid lineage. CML progresses through three successive stages. Chronic phase chronic myeloid leukemia (CML-CP) can last for years, which continues through an accelerated phase en route to a blast crisis, resembling acute myeloid leukemia or lymphoid leukemia. Within the blast crisis stage, the patient survival time is under a year (Assouline and Lipton, 2011).

The Philadelphia chromosome (Ph) was discovered in 1960 followed by its detailed cytogenetic characterization in 1973 (Nowell and Hungerford, 1960; Rowley, 1973). Translocation of chromosomes 9 and 22 results in the de novo formation of the BCR-ABL fusion oncogene, a constitutively active form of the ABL tyrosine kinase. BCR-ABL kinase hyperactivity enhances proliferation and growth-factor independence while reducing apoptosis (Jabbour et al., 2010; Zhang and Rowley, 2011). The molecular characterization of CML provided rationale for studying all cancer at the gene level and has influenced the entire field.

The mutant BCR-ABL kinase represents a specific cancer target not shared with normal somatic cells, and it was reasoned that, if this specific cancer gene could be targeted, the cancer could be cured. In an effort to inhibit the activity of the BCR-ABL kinase, a small-molecule compound now referred to as imatinib was developed (Druker et al., 1996, 2001). Imatinib blocks the ATP-binding site of BCR-ABL, suppressing kinase signaling and inducing cell death. The results of imatinib therapy are impressive for CML-CP patients, with a 7-year overall survival rate of 86% (Jabbour et al., 2010). Imatinib is currently the recommended first-line therapeutic for CML-CP patients and is accepted as the standard of care.

The overwhelming, inspiring success of imatinib has become the example to follow for cancer research, providing the key rationale in favor of various cancer genome sequencing projects. Vast investments in high-throughput genome sequencing technologies and microarray analyses have resulted in the identification of many candidate molecular targets. Unfortunately, molecular targeting success of this magnitude has yet to be repeated for the majority of solid tumors (Heng et al., 2010b). Identifying recurrent chromosomal changes has proven to be extremely challenging in solid tumors due to the lack of recurrent patterns in most tumor types coupled with a high level of non-clonal chromosome aberrations (NCCAs) and karyotypic heterogeneity (Heppner and Miller, 1998; Albertson et al., 2003; Heng et al., 2004, 2013). The vast majority of gene mutations are not shared among patients, and overwhelming mutational heterogeneity can occur within a tumor (Bielas et al., 2006; Heng, 2007; Ye et al., 2007; Navin et al., 2011). Furthermore, even when a recurrent mutation is present, as in the case of BRAF mutations in melanoma, the effect of a targeted drug such as vemurafenib is dramatic but transient, as tumors invariably become resistant to these agents (Wagle et al., 2011). To understand why the high success of molecular targeting against CML-CP has been difficult to duplicate for most solid tumors, we analyze the following issues.

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BOX 1. Select Terminology and Definitions

Term	Definition
Genome	The entity that contains an organism's hereditary information (system inheritance), represented by both gene context and genomic topology
	The topology of the genome provides the physical basis of genomic architecture and a provides the physical basis of genomic architecture and multi-dimensional interactive relationship that exists between all genes and non-coding sequences
	The genome is the main evolutionary selection platform
Punctuated phase	Phase of NCCA/CCA cycle that is marked by genome replacement coupled with elevated non-clonal chromosome aberrations
Stepwise phase	Phase of NCCA/CCA cycle that is marked by clonal evolution with clonal chromosome aberrations, where stepwise Darwinian evolution is dominant
NCCA/CCA cycle	Highly dynamic and stochastic pattern of solid tumor evolution
	Consists of a punctuated phase and a stepwise phase
	Shifts between phases are induced by stress and subsequent selection
	Progression during the punctuated phase cannot be traced, unlike in the stepwise phase

Comparative Analyses

Contrasting patterns of evolution

Cancer represents an evolutionary process, where the pattern has been demonstrated to be highly dynamic and stochastic (Merlo et al., 2006; Gatenby et al., 2009b, 2010; Gillies et al., 2012). In particular, solid tumor evolution is cyclical and consists of two distinct phases: a punctuated phase (marked by genome replacement coupled with elevated NCCAs) and a stepwise phase (marked by clonal evolution with dominant clonal chromosome aberrations or CCAs) (Heng et al., 2006a,c, 2011a,b; Heng, 2007, 2013) (Box 1). Shifts between phases are induced by stress and subsequent selection. Recently, this discontinuous pattern of evolution has been supported using single-cell sequencing. At the DNA-level, tumors grow by "punctuated clonal expansion with few persistent intermediates" (Navin et al., 2011). Even if the stepwise phase is detected at the DNA level (which represents the building materials within the genome network), the punctuated phase may persist at the genome level (which represents network architecture), as the same building materials can be utilized to build different structures. Therefore, the key to monitoring cancer evolution is at the genome level rather than the gene level (Heng et al., 2011a).

Discovery of the two phases of cancer evolution is of clinical significance, as specific molecular targeting is most effective within the stepwise phase of evolution, but less useful in the punctuated phase where there are no fixed targets. During the punctuated phase, the genetic landscape of a tumor can drastically and rapidly change, and alterations of the genome network can severely impact drug efficacy.

Based on the key clinical characteristics of CML, its evolutionary pattern resembles the stepwise model of evolution. In fact, the clonal evolution hypothesis is supported by the case of CML and has formed the conceptual framework for current cancer research (Nowell, 1976). Failure of this applied model in tumors has been a continuous source of frustration, especially since solid tumors represent 90% of all malignancies.

The evolutionary process of solid tumors does not fit the gradual linear pattern observed in CML. Most solid tumors are marked by the universal existence of genome heterogeneity, where tumors of the same type often contain unique karyotypes and mutations found within subclones (Heppner and Miller, 1998; Heng et al., 2004, 2006a; Losi et al., 2005; Merlo et al., 2006). High-throughput sequencing has recently confirmed this (Gerlinger et al., 2012). Therefore, it is necessary to illustrate the contrasting patterns between clonal evolution (CML) and stochastic evolution (most solid tumors), which represent the basis behind the failure of applying the targeting success of CML-CP to most solid tumors.

It is important to illustrate why CML and other solid tumors display different patterns of somatic cell evolution. Reviewing the system features and behaviors of CML-CP and prostate cancer reveals the following three key evolutionary characteristics that contrast CML from most solid tumors: fusion gene dominance, temporal order of karyotypic evolution, and causation of cancer progression by a highly penetrant fusion gene. Prostate cancer represents an example of a solid cancer that is the focus of extensive fusion gene research (Tomlins et al., 2005, 2008; Rajput et al., 2007; Tu et al., 2007; Rubin et al., 2011). In particular, the identification of the fusion gene TMPRSS2-ERG in prostate cancer samples has reinforced the hope that a molecular Achilles' heel exists within every cancer.

Fusion gene dominance

CML is characterized by the high-penetrance of the BCR-ABL fusion gene. In most instances, the typical t(9;22) is the sole chromosomal aberration during chronic phase (Johansson et al., 2002). Prostate cancer cases, however, are marked by high karyotypic heterogeneity, and specific single fusion genes occur in reduced frequencies within the patient population. Dozens of chromosomal abnormalities and fusion genes have been identified in prostate cancer cases (Gu and Brothman, 2011), suggesting the involvement of large cohorts of genes and chromosomal aberrations. This is in contrast to the single fusion gene culprit characteristic of CML-CP. A recent study demonstrated the presence of the extensively studied fusion gene TMPRSS2-ERG in only 46% of prostate cancer biopsies (Mosquera et al., 2009). In fact, after comparing published data (28 studies totaling 2,786 patient samples, detailed analysis not shown), the range of TMPRSS2-ERG fusion gene occurrence in prostate cancer patient samples is approximately 15.3–77.8%, with a mathematical average of 42.3%. This suggests that even though fusion genes may be involved in solid tumor progression, the penetrance of the gene products is very different from the high frequency found in CML-CP. It is important to note that, despite the application of large-scale genome sequencing, commonly shared fusion genes have not been identified for most solid tumors.

Temporal order of karyotypic evolution

Despite the high level of additional chromosomal changes detected from the majority of CML patients in blast crisis, along with variance in the temporal order of secondary changes, the preferred pathway appears to start with i(17q), followed by +8 and +Ph, and then +19, suggesting a stepwise pattern of karyotypic evolution from chronic phase to blast crisis (Johansson et al., 2002). Corresponding to the karyotypic changes, the over-expression of the BCR-ABL fusion gene, up-regulation of the EV11 gene, increased telomerase activity, and mutation of RB1, TP53, and CDKN2A have been documented.

A distinct common order of karyotypic evolution has not been characterized in prostate cancer. In contrast, the genomic rearrangements studied in prostate cancer do not occur in a predictable fashion. In a recent paired-end, massively parallel sequencing project of seven prostate cancer patients (Berger et al., 2011), three of the seven tumor samples sequenced were positive for TMPRSS2-ERG rearrangements. Interestingly, but not surprisingly, the sequencing results suggested that TMPRSS2-ERG rearrangement positivity of each sample was

the result of a unique pattern of complex chromosome breakage and rejoining. This supports the discontinuous pattern of solid tumor evolution (Heng et al., 2006a, 2010a), and one would expect a large number of possible chromosomal rearrangement patterns that result in TMPRSS2-ERG rearrangement-positivity.

Causation of cancer progression by a highly penetrant fusion gene

The hyperactivity of the BCR-ABL kinase has been deemed the force driving cells from chronic phase to blast crisis due to its involvement in enhanced proliferation, growth-factor independence, reduced adhesion of tumor cells, and reduced apoptosis. This is supported in mice transgenic for a BCR-ABL p190 DNA construct (Heisterkamp et al., 1990). Of the 10 transgenic mice generated, 8 died or were moribund with acute or chronic leukemia, myeloid or lymphoblastic, between 10 and 58 days after birth. Two of these were diagnosed in the blast crisis of CML. In prostate cancer, however, fusion genes have not been demonstrated as the driving force of disease progression in vivo. Transgenic overexpression of ERG in mice resulted in the development of prostatic intraepithelial neoplasia, but these lesions did not progress to invasive prostate cancer (Klezovitch et al., 2008; Tomlins et al., 2008).

Population structure of hematologic and solid cancers

A review of population genetics further contrasts hematologic and solid cancers (Table I). Population size plays an important role in shaping the evolutionary patterns. Cell populations of hematological malignancies occupy a large blood environment. Within this system, initially altered cells can freely move. Any dominant alteration, such as the appearance of fusion gene products, would have a significant impact on the entire system. According to population genetics, clonal events within a large population can be dominant over non-clonal events (Gerrish and Lenski, 1998). In contrast, altered cells in solid tissues are constrained by tissue geography and local micro-environments are different, unlike the tightly regulated, relatively uniform blood environment. These altered cells represent typical small, isolated populations.

Small population size implies that genetic drift has a greater influence on evolution. Solid tumors, which represent isolated small populations, mediate their evolution through the NCCA/CCA cycle (Heng et al., 2006a). NCCAs develop into different CCAs in different tumors due to the influence of genetic drift. This principle has also been discussed in regard to the correlation between dominant mutation types, the size of a tissue within a cellular compartment, and the size of a stem cell pool (Frank and Nowak, 2004). Tissue compartments with large stem cell pools often incur rapid cellular proliferation caused by tumor suppressor and oncogene mutation, whereas small stem cell pools may often initiate cancer progression via genetic instability (Frank and Nowak, 2004). A direct link between NCCAs and genomic instability was found after observing elevated frequencies of NCCAs of various cell lines and animal models carrying defects in genes responsible for maintaining genetic diversity (Heng et al., 2006b,c, 2009, 2011a). On the other hand, CCAs are associated with dominant pathways, which explains the dominance of fusion genes in the large population blood cancers and the heterogeneity of aberrations detected from the small and isolated population solid tumors. As a result, the evolutionary process of these different isolated populations is diverse, requiring a longer time to evolve due to additional system constraint.

Comparing different stages of disease progression

As CML patients progress from the chronic phase into the accelerated and blast crisis stages, imatinib efficacy plummets. Complete cytogenetic response in early chronic phase patients placed on imatinib is approximately 80%. This falls to ~8% in blast crisis (Radich, 2007), where the median survival time is measured in months (Assouline and Lipton, 2011). This compares to the efficacy of EGFR targeting in prostate cancer, as monotherapy agents have failed to demonstrate high antitumor activity in clinical trials (Canil et al., 2005; Gravis et al., 2008; Guérin et al., 2010; Sridhar et al., 2010).

The frequency of additional chromosomal abnormalities increases with progression in CML. This frequency is ~7% in chronic phase patients and jumps to 40–70% in the advanced stages (Skorski, 2011). These advanced stages of the disease resemble the majority of solid tumors, where the increase of genomic instability and accumulation of genetic changes are key features that are age-related and are responsible for a relatively longer time period for the cancer to develop and progress. The linkage between genomic instability and poor prognosis has been well documented in both hematologic and solid cancer patients (Nishizaki et al., 2002; Nakamura et al., 2003; Caraway et al., 2008; Sato et al., 2010; Zamecnikova et al., 2010).

We then suggest that with imatinib, we are actually treating a stage of CML that is comparable to the benign phase of solid tumors. Unfortunately, while a dominant CML-CP signature (BCR-ABL) has been identified with cytogenetic techniques, a dominant specific fusion gene that drives cancer progression has yet to be identified in prostatic benign tissue. If a dominant fusion gene was present in benign tissue that acted as a driving force in the progression of solid tumors, clinicians could identify threatening tissues before they became problematic. Similar to the elimination of chronic phase leukemic cells with imatinib, extraction of these threatening benign tissues conceptually would be much more effective than current treatments on late-stage solid tumors. Unfortunately, this is clearly not the case in prostate cancer progression.

TABLE I. Evolutionary characteristics of hematologic and solid cancers

Feature	Hematological malignancies	Solid cancers
Cell population size	Large population size	Small population size
Cell motility	Cells are free to migrate throughout blood environment	Cells populations are isolated and constrained by tissue geography
Genetic drift	Lower influence on large populations	Greater influence on small populations
Micro-environment	Blood stream is tightly regulated and relatively uniform (glucose and oxygen levels, pH, etc.)	Micro-environments vary widely within and between tissues
Cell metabolism	Influenced by normoxic conditions, regulated nutritional levels	Varies depending on normoxic/hypoxic conditions and nutritional gradients
Drug delivery/targeting efficiency	Free motility of cells allows for optimal drug targeting	Varying environments may affect drug chemistry, stationary tumor masses of cells potentially hinder drug targeting and penetration
Cell lineage of disease onset	Early lineage displays more defined differentiation and is characterized by orderly and more predictable stages	Late lineage displays less linear progression and is characterized by stochastic, unpredictable stages

Conclusions

Molecular targeting success is, unfortunately, very limited in other cancer types where the evolutionary patterns are significantly different. While CML-CP clearly represents a stepwise model, most detectable solid tumors likely have undergone multiple rounds of the two-phase cycle of evolution. The associated genome dynamics make it very difficult to successfully apply molecular targeting approaches against most solid tumors. Furthermore, if one particular pathway of a solid tumor is blocked by a specific therapy, genomic instability can relieve the requirement for that pathway within the population of surviving cells. This is evidenced by the demonstration that drug treatment can induce the recently introduced phenomenon of genome chaos, where major genome reorganization is achieved in a short period of time following chromosome fragmentation (Heng et al., 2006c, 2011b; Stevens et al., 2007, 2011a). This view is in agreement with the clinical observations that CML responds to imatinib more effectively during the chronic phase than during blast crisis where new karyotypic aberrations are detectable.

Despite attempts to apply molecular targeting principles to solid tumors, clinical outcomes have been far from ideal. Metastatic melanoma patients treated with vemurafenib (targeting the BRAF V600E mutation) for 6 months had a 20% overall survival increase compared to dacarbazine treatment (Chapman et al., 2011). However, between the 9- and 10-month mark, the overall survival trends of these two treatments appear to converge, suggesting that vemurafenib may prolong the survival of metastatic melanoma patients by approximately 2 months. A 4-year study regarding trastuzumab as part of an adjuvant treatment regimen against HER2-positive breast cancer concluded that patients treated with trastuzumab for 1 year had an overall survival increase of 1.6% over those observed without treatment (Gianni et al., 2011). Such cases do not mimic the overwhelming success of imatinib.

Studies of 82 non-small-cell lung cancer (NSCLC) patients treated with crizotinib targeting the EML4-ALK fusion gene observed 1- and 2-year overall survival rates of 74% and 54%, compared to 1- and 2-year overall survival rates of 72% and 36% of ALK-positive crizotinib-naïve control patients (Kwak et al., 2010; Shaw et al., 2011). Based on previous data from targeted therapy trials of other solid tumors, one would expect further decline in survival as this study continues. A recent meta-analysis of 13 randomized trials evaluating the effects of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (erlotinib and gefitinib) in 1,260 patients with EGFR-mutated NSCLCs concluded that despite a higher response rate than platinum-based chemotherapy (67.6% vs. 32.8%, respectively), EGFR tyrosine kinase inhibitors do not significantly improve the overall survival of patients compared to control groups (hazard ratio = 0.96; Petrelli et al., 2012). These studies indicate, unfortunately, that the molecular targeting success against CML-CP has not been replicated in the clinic against most solid tumors.

Interestingly, treating PML-RARA-positive acute promyelocytic leukemia (APL) patients with a combination of arsenic trioxide and all-trans retinoic acid has been very successful, with a 5-year overall survival rate of 97.4% (Hu et al., 2009). This is not surprising considering the parallels between APL and CML-CP where both are typically characterized by a highly penetrant, dominant fusion gene (PML-RARA is found in over 98% of APL cases) (Vitoux et al., 2007). In contrast, the fusion gene EML4-ALK is found in only 4% of NSCLC cases (Shaw et al., 2011). Like BCR-ABL mouse models, PML-RARA expression yields APL in transgenic mice (de The and Chen, 2010), demonstrating the direct link between the fusion gene and the onset of the disease. Both diseases are hematological malignancies with similar population structures, ultimately allowing for a dominant alteration (e.g. fusion gene) to have a significant impact on the entire system. It is therefore likely that some subtypes of cancer could be effectively treated using target-specific or even less specific therapy, during the stepwise phase of cancer evolution, when they have evolutionary patterns similar to those of CML and APL. Of course, molecular targeting can further reduce potential side effects otherwise associated with harsh, general cellular mechanism-focused treatment such as chemotherapy.

The application of fusion genes in the diagnosis of solid tumors also has limited implications due to the inaccessibility of threatening benign tissues using current sampling techniques. A simple, accurate, and informative blood draw can be performed to diagnose patients with BCR-ABL positive chronic phase leukemia due to the constant circulation of leukemic cells in the blood stream. However, fusion gene identification in threatening benign tissue within an asymptomatic individual is problematic. Current biopsies collect only a small sample of suspect tissue. Even if the sample contains altered tissue, the biopsy will not likely indicate the complete genomic profile of the tumor, given the vast genomic heterogeneity associated with solid tumors. This is unfortunate because if we could identify solid tumors in the benign phase, for many cases, surgical resection would be sufficient even without drug treatment.

This comparison also sheds light on the concept of oncogene addiction, where tumor maintenance is dependent on the constitutive activity of oncogenes, and inhibition of this activity leads to tumor cell death, differentiation, arrest, or senescence (Luo et al., 2009). This concept is supported by a BCR-ABL1-tetracycline transactivator double transgenic mouse study (Huettner et al., 2000). Reversion of the leukemic phenotype and complete remission were achieved after suppression of the BCR-ABL1 gene. However, this concept fails to extend to most types of cancer due to the lack of a dominant gene product that drives cancer progression in early lineages. In addition, any oncogene addiction can be lost to subsequent rounds of the NCCA/CCA cycle, resulting in system-wide changes that can impact target-specific drug resistance without necessarily resulting in additional mutations to the target gene product. This explains the loss of oncogene addiction seen in lung cancer, breast cancer, as well as the blast crisis of CML, despite the expression of targetable EGFR, HER2, and BCR-ABL, respectively (Hochhaus et al., 2002; Sharma et al., 2007; Valabrega et al., 2007). We can confirm that under only very rare, special circumstances does this model of oncogene addiction actually apply to cancer.

Imatinib-resistant CML cases have been attributed to point mutations in the BCR-ABL gene, however, these mutations are actually found in only a small subset of imatinib-resistant BCR-ABL CML cases (Deininger et al., 2005). A recent study of the apoptotic machinery of BCR-ABL-driven leukemia suggested that the complexity of the disease clearly extends beyond any point mutations that may occur within the kinase as cases with higher resistance actually involve additional genomic changes rather than new kinase point mutations (Kaufmann, 2006). Our recent study of cell death heterogeneity may explain this problem. Since the cell death process can also favor cancer evolution by changing multiple levels of genetic and epigenetic organization, there are many off-target and adverse effects (Stevens et al., 2013). Extension of the fusion gene target model derived from CML-CP to solid tumors will be ineffective due to the even greater complexity and heterogeneity within these diseases, therefore, we can no longer follow CML's lead in the design of future cancer research.

What is the new direction we should take in the war against cancer, since specific molecular targeting has not been an ideal approach for most solid tumor types due to overwhelming genome instability in most solid tumors? A new, promising

strategy involves treating cancer progression as system evolution, where focusing on the overall pattern of system evolution rather than targeting individual genes may provide the answer (Heng et al., 2006a,b,c, 2010a, 2011a; Gatenby et al., 2009a,b, 2010; Gillies et al., 2012). One established system of using NCCAs to study karyotypic heterogeneity and monitor the speed and phases of cancer evolution represents such an example (Heng et al., 2009; Ye et al., 2009; Stevens et al., 2011b; Heng, 2012). The key here is to constrain the speed of tumor growth without triggering genome chaos, which promotes the emergence of aggressive, drug-resistant tumor subpopulations. Targeting specific pathways works well only when the system is stable, during the stepwise phase, however, for unstable systems, pathway targeting is quickly overcome by the evolution of the system. Even worse, through genome chaos, new pathways are selected and constructed, and new genomes (systems) are rapidly formed. Therefore, drug intervention can, in fact, paradoxically promote cancer evolution when applied in the wrong phase (Maley et al., 2004). In contrast, slowing the evolutionary process by carefully constraining the system without promoting genome chaos will improve patient prognosis (Heng, 2013).

The main purpose of our analysis is not just to be critical of current efforts, nor to offer precise solutions, but to call upon investigators to actively discuss this important issue, which is crucial for our future efforts towards winning the war on cancer. Such action is urgently needed, as there are currently two opposite viewpoints when dealing with this question. On one side, is that it is well known that imatinib represents an exception, but without a clear explanation. Paradoxically, other researchers believe that, with continued efforts, the success of imatinib in CML-CP will be duplicated in most solid tumors. With this evolutionary analysis, we hope that we have done the first key step by challenging the research community to face this reality and adopt a new understanding of cancer. With the correct conceptual framework, we can make the next triumph of cancer research (Heng, 2007, 2012, 2013; Heng et al., 2011a,b).

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Evolutionary Mechanism Unifies the Hallmarks of Cancer

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Mini Review

The basis for the gene mutation theory of cancer that dominates current molecular cancer research consists of: the belief that gene-level aberrations such as mutations are the main cause of cancers, the concept that stepwise gene mutation accumulation drives cancer progression, and the hallmarks of cancer. The research community swiftly embraced the hallmarks of cancer, as such synthesis has supported the notions that common cancer genes are responsible for the majority of cancers and the complexity of cancer can be dissected into simplified molecular principles. The gene/pathway classification based on individual hallmarks provides explanation for the large number of diverse gene mutations, which is in contrast to the original estimation that only a handful of gene mutations would be discovered. Further, these hallmarks have been highly influential as they also provide the rationale and research direction for continued gene-based cancer research. While the molecular knowledge of these hallmarks is drastically increasing, the clinical implication remains limited, as cancer dynamics cannot be summarized by a few isolated/fixed molecular principles. Furthermore, the highly heterogeneous genetic signature of cancers, including massive stochastic genome alterations, challenges the utility of continuously studying each individual gene mutation under the framework of these hallmarks. It is therefore necessary to re-evaluate the concept of cancer hallmarks through the lens of cancer evolution. In this analysis, the evolutionary basis for the hallmarks of cancer will be discussed and the evolutionary mechanism of cancer suggested by the genome theory will be employed to unify the diverse molecular mechanisms of cancer.

The Significance and Limitations of Using the Hallmarks of Cancer to Understand the Complexity of Cancer

The majority of current cancer research is based on the gene-centric view, which promotes the identification of shared gene mutations in cancer cells as well as focuses on distinguishing individual molecular pathways responsible for the initiation and progression of the disease. Hundreds of cancer genes have been identified as a result of decades of research, and the characterization of these genes has generated large numbers of hypotheses and publications. However, synthesis of these diverse and often conflicting molecular data has been a challenge.[1,2] The large number of identified cancer gene mutations has significantly surpassed the original prediction of the gene mutation theory that there should be only a limited number of key cancer gene mutations.[3] To understand the common biological basis for this substantial number of gene mutations and to reconcile the inconsistencies between theoretical prediction and clinical fact, various biological capabilities and enabling characteristics of cancer that are believed to facilitate tumor growth and metastasis have been summarized in order to categorize all cancer genes and their molecular contributions to cancer. These distinctive features, termed hallmarks, were concisely explained as the six common traits that direct the transformation of normal cells to malignant cells. They include growth stimulation, evasion of growth suppressors, resistance of apoptosis, replicative immortality, induction of angiogenesis, and activation of invasion and metastasis.[4] This synthesis was immediately accepted by the research community with great enthusiasm, reflected by an extremely high number of citations, as it not only provides the rationale for the search of more cancer genes but also points to the direction of where to look for them. Recently, abnormal metabolic pathways and evasion of the immune system were added to the list of hallmarks, along with the enabling characteristics of genome instability and tumor-promoting inflammation.[5] In short, cancer cells are thought to emerge as the result of the accumulation of defects in the control mechanisms of cell division and regulatory feedback systems. These mechanisms are controlled by specific genes and pathways. One example is the TP53 tumor suppressor protein that normally triggers cell death in response to DNA damage. A mutation of the gene would inhibit its monitoring capabilities of the apoptosis-inducing

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circuitry and enable cancerous cells containing damaged DNA to resist programmed cell death.[5] Thus, the complexity of cancer is simplified into a short list of underlying principles that can be effectively explained by key cancer gene mutations or genetic network interactions. Coupled with the general concept that cancer progression follows stepwise, clonal evolution driven by several of these key mutations,[5–7] and the established relationship between gene mutation and causality of cancer in experimental settings, the hallmarks of cancer have further cemented the gene mutation theory of cancer.

With these accepted frameworks, cancer research should be straightforward. Identifying gene mutations responsible for these hallmarks should establish the molecular mechanism of cancer, and the next logical step would be application of this mechanism both in diagnostic and treatment regimens. However, the reality of cancer is far more complicated, and too many implications that are based on studying these hallmarks have failed in the clinic. It is now time to re-examine the concept of the cancer hallmark itself.

Indeed, there are conceptual challenges to the hallmarks of cancer. First, both the number of hallmarks and identified gene mutations are expanding. On one hand, there are a large total number of genes that can be linked to these hallmarks. In particular, due to the current cancer genome sequencing project, the list of cancer gene mutations has grown rapidly, making the hallmark concept less clinically relevant. On the other hand, to make the most sense of these newly identified gene mutations, the list of hallmarks must also increase, as the majority of gene mutations cannot be explained by the current hallmarks. Surely, the number of hallmarks has increased from six to ten (since these enabling characteristics of genomic instability and tumor-promoting inflammation are hallmarks, and in fact, genomic instability perhaps is the most important hallmark both for cellular heterogeneity and unifying other hallmarks.). Furthermore, as human behavior and environmental setting are associated with survival rate of cancer patients, these types of higher-level system constraints will likely increase the number of hallmarks. Further, more specific mechanisms linked to cancer such as viral infection (e.g. human papillomavirus) would also qualify as additional hallmarks. For complex systems, small increases of the number of involved agents will lead to huge complications.

This raises some important questions. At which point does the list become comprehensive, where no additional hallmarks are necessary? Which hallmarks are more important than others (*i.e.* the hallmark of the hallmarks)? How do we prioritize these hallmarks in the clinic (*i.e.* which do we target first)? If each hallmark of this long list holds equal importance, the rationale of establishing the hallmarks in the first place would be lost. Even though the importance of each hallmark is not formally discussed in the literature, many researchers assume these hallmarks are more or less equally important. No one has even admitted that the hallmark they are studying is less important. Quite the opposite, everyone would consider the hallmark they study the most important. Therefore, this issue needs to be discussed, as there are many conflicts among hallmarks.

Second, there is a high degree of overlap and dynamics for these hallmarks. A hallmark may require additional hallmarks, for example metastasis incorporates cell death, proliferation, inflammation, metabolism, and avoiding immune destruction.[8,9] In addition, the same gene can be involved in different hallmarks. Mutant *RAS* and upregulated *MYC* play roles in energy metabolism, proliferative signaling, angiogenesis, invasion, and survival.[5,10–14] Regulation of the well-characterized metastatic suppressor *KISS1* has also been linked to tumor metabolism, affecting glycolysis, mitochondrial biogenesis, and lipid homeostasis.[8] Telomerase and its protein subunit TERT, commonly associated with elongation and maintenance of telomeric DNA, exert telomere-independent functions including enhancement of cell proliferation and/or resistance to apoptosis, involvement in DNA damage repair and RNA-dependent RNA polymerase function, and amplifying signaling of the WNT pathway by serving as a cofactor of the β-catenin/LEF transcription factor complex.[5,15–19] This overlap makes the combinational function of many genes and gene mutations difficult to predict. If a tumor generates its own proliferative signal, but still responds to antigrowth and cell death signaling, how does the cell population respond? Furthermore, not only can the hallmarks conflict with each other, but even the same hallmark can be either beneficial or harmful for a given cancer within the changed evolutionary landscape. For example, the process of effective cell death can eliminate most cancer cells, but also can accelerate cancer evolution by promoting outlier-driven repopulation, resulting in a more aggressive phenotype (Horne *et al.*, unpublished observations).

Third, it is also important to note that the hallmarks of cancer are not all unique to malignant tumors. When comparing malignant and benign tumors, only one of the six original hallmarks, tissue invasion and metastasis, is not shared by both.[20] Thus, it appears less than ideal to continue placing all ten, supposedly distinctive capabilities on par with each other, blurring the lines between the designation of benign tumors and cancer.

Fourth, many linkages established between gene mutations and these hallmarks have been based on experimental systems where average profiling of cell populations was performed.[20] Since molecular averaging methods artificially "wash away" cellular heterogeneity (either by using more homogenous cell lines or by specifically profiling the dominant populations by ignoring large portions of cells that exhibit non-clonal changes), it is much easier to establish a specific "average linkage." In addition, much molecular characterization of individual hallmarks is focused on specific linear pathways. Unfortunately, tumors generated in the laboratory setting within artificial conditions, usually driven by dominant, linear pathways, differ from those in

patients that have undergone decades of evolution. This explains why many conclusions do not truly reflect a "real world" cancer cell population where the heterogeneity and complexity rule. It was recently demonstrated that, in most cancer cell populations that exhibit unstable genomes, there is no average profile.[21] For a given cell population, it will often display different types of hallmarks. Importantly, many hallmarks represent population-level emergent behaviors, such as angiogenesis, and therefore may not be dissected down to individual cell-based explanations. Recently, some criticism has focused on the hallmarks concept, in particular by linking hallmarks to cell culture artifacts in addition to the overall framework.[22]

Fifth, understanding the molecular basis of these hallmarks and identifying genes for each hallmark has limited clinical relevance. While it is well known that inflammation represents a new hallmark, a recent clinical study showed that inflammation status is associated with reduced risk of prostate cancer.[23] A similar limitation is best illustrated in cancer treatment. While most of the molecular targets are well-characterized, in real tumors, they quickly become moving targets under medical treatments, especially when the treatment pressure is high, rapid and massive genome reorganization (termed genome chaos) occurs, which can drastically change dominating pathways.[24–26] Interestingly, mathematical and evolutionary modeling have supported that therapeutic intervention can provide selective pressure for the expansion of resistant variants,[27,28] and treatment has been proposed to accelerate cancer genome evolution and tumor progression.[29] This would suggest that, regardless of which hallmark is targeted, the cancer adapts and persists. While the gene theory has been the foundation of a sizable quantity of experimental findings and molecular progress, there is a huge gap between understanding how a molecular mechanism potentially contributes to cancer and how cancer occurs in reality, and whether it is practical to apply that knowledge in the clinic. For example, this gap becomes highly significant as physicians aim to increase patient survival and reduce tumor cell burdens, while researchers are focused on identifying molecular mechanisms based on simplified models. Clearly, just focusing on individual hallmarks is not going to offer realistic understanding and treatment options for most cancers. The truth is many individual hallmarks represent emergent properties, which are difficult to directly link to genetic parts, or individual cells, or even subpopulations (Table 1).[30,31]

Lastly, the hallmark concept seemed to have rationalized and energized the field of cancer research, comforted researchers[20] and suggested the importance of focusing on cancer phenotypes. It in fact failed to address the fundamental issue of the dynamic relationship among cancer phenotypes throughout the evolutionary process, as cancer cells are constantly evolving and the effort to dissect the highly dynamic phenotypical package into individual molecular principles often ignores the evolutionary context. For example, the concept helps to understand why many gene mutations can potentially contribute to cancer by linking them to individual hallmarks, but did not explain their underlying evolutionary mechanism, nor provide the framework to understand why there are so many mutations detected yet the prediction power of an individual gene mutation is low in the clinic. Furthermore, they failed to appreciate the ultimate importance of the genome's stochastic dynamics and the genome's role to organize gene mutations and serve as a package for macro-cellular evolutionary selection as well as the dynamic competition and collaboration within cellular populations. Obviously, if there were a small number of cancer gene mutations like the previous prediction of less than a handful, cancer cell populations were homogeneous, and if each hallmark were truly independent from each other, then the established linkage between cancer genes and hallmarks would be highly significant. However, as illustrated by the current cancer genome sequencing project, there are so many gene/epigene mutations/alterations, the cancer genetic landscape is highly diverse, and there is no one-to-one relationship between gene mutations and hallmarks. Thus, ongoing efforts to link each newly identified gene mutation to these hallmarks might be useful for publication purposes or convincing funding agencies, but offer limited clinically useful information. More significantly, the hallmark concept is not a theory of cancer, and it does not provide the theoretical understanding of how and why we get cancer.

To address these issues, a unified or general theory is urgently needed,[43,44] which represents the common mechanism behind the hallmarks of cancer and provides the explanation for the large number of diverse genetic and epigenetic alterations observed. Since cancer represents a typical evolutionary process,[7,35,42,45] it is logical to apply evolutionary theory to achieve this goal. One such framework that takes a systematic approach and can rectify these disparities is the genome theory of cancer evolution. During the revision of this mini-review, Dr. Robert Weinberg published his candid analysis of current cancer research, and despite the knowledge of the hallmarks and their molecular understandings, he clearly admitted that there is no correct framework of cancer research.[46] Thus, the original idea of searching for this framework by molecular principles is not working. Interestingly, the authors of the cancer hallmark concept did not even anticipate that this would become so popular, perhaps due to some of the above concerns!

Genome Theory of Cancer Evolution Offers a Genome System Approach and Unifies the Hallmarks of Cancer

The genome theory of cancer evolution was introduced to establish a new theoretical basis of current cancer research (Table 2). At the center of this theory is the redefinition of the genome and its relationship with genes in the contexts of genetic inheritance and somatic cell evolution. There are an

Table 1. Explanations of key terminologies

Clonal and non-clonal chromosome aberrations:
Current cytogenetics defines a clonal chromosome aberration (CCA) as a given chromosome aberration which can be detected at least twice within 20 to 40 mitotic figures, while a non-clonal chromosome aberration (NCCA) is observed at a frequency less than 4% (less than 2 in 50 mitotic cells examined).[32] Researchers focus on CCAs and dismiss NCCAs. The function of NCCAs has recently been revisited based on its linkage with genome instability.[9,32–34] NCCAs/CCAs represent the evolutionary trade-off between survival and proliferation. The potential confusion comes from the complicated relationship between linkage and gene/genome concept. The term "clonal" has two meanings: lineage and identity (or similarity). Although a clear lineage may be determined based on historical information and/or short sequences of DNA, it does not mean that the cells share the same genome. For example, parental and daughter cells are connected by lineage, but in most cancers, parental and daughter cells often display different genome identities. In contrast to the traditional assumption that genome-level change would be passed on to the daughter cells if the cell divides, for these cells with unstable genomes, parental cells cannot pass on the same genome.[25,32,35,36] This leads to the unique feature of the cancer cell population, where an entire cell population can display different types of genomes.
Punctuated and stepwise somatic cell evolution:
Punctuated and stepwise evolution initially referred to the karyotype pattern observed in an immortalization model where both non-clonal and clonal expansion were detected.[33] Now, these concepts also apply to the DNA level, as sequencing efforts have recently confirmed these evolutionary phases in cancer progression.[37–39] Different from clonal diversification, there are massive infrequent chromosomal aberrations within the punctuated phase leading to stochastic genome re-organization, interrupting the inheritance of karyotypes between mother and daughter cells. In the clonal phase, however, the majority of cells are clonal across generations with traceable karyotype diversification. Punctuated equilibrium was proposed to explain why most species exhibit minimal net evolutionary change (phenotype) for most of their geological history, and significant evolutionary changes occur rarely and rapidly (on a geologic time scale). We borrowed the term punctuated to describe the rapid and drastic genome-level changes in contrast to stepwise evolution where the same genome is maintained coupled with possible gene mutation accumulation. As illustrated in Figure 1, we use "punctuated phase" to distinguish from the gene-mediated clonal expansion and diversification phase.[32–34,40]
Macro-cellular evolution and micro-cellular evolution:
Macro-cellular evolution refers to karyotype change-mediated somatic cell evolution, whereas micro-cellular evolution refers to gene/epigene change-mediated evolution where the karyotype remains the same. Macroevolution and microevolution respectively refer to the organismal evolution at the above-species level or at the population level within a species. To distinguish the cancer evolutionary process based on the contribution of genome or gene, and punctuated or stepwise pattern, we use macro- and micro-cellular evolution.[25,33,35,41] In the concept of species, individuals share the same karyotype. For individual cancer cells, they can or cannot share the same karyotype. In addition, macro-cellular evolution represents system replacement while micro-cellular evolution represents the modification of the genome-defined system.
Multiple levels of emergent properties:
Since cancer is a disease of tissue and higher-level organization rather individual cells alone, the multiple levels of emergent properties refer to the non-deterministic relationship between higher-level system order and interaction among lower-level components. At the individual cell level, self-organization is based on the genome. At the cell population level, there is no simple average property due to the heterogeneity of the cell population. At the microenvironmental interaction level, large numbers of cell populations of distinct cell types will be involved. It is extremely hard to treat this dynamic disease using isolated phenotypes. To explain these emergent properties, we recently have identified yet another new type of inheritance, inherited heterogeneity, to explain how heterogeneity emerges. It is likely that self-organization and evolution are two very powerful interactive forces that shape the bio-system, and evolution plays an important role to select and maintain potential emergent properties.[42]

increasing number of diseases that have been linked to the concept of evolutionary adaptation at the somatic cell level.[9,40] It is thus timely to illustrate how somatic cell evolution works. Using the genomic-evolutionary perspective, cancer is described as a genome system-level disease displaying altered genomes coupled with increased heterogeneity and other genetic and phenotypic complexities. In other words, cancer evolution can be understood as a series of genome-mediated system replacement involving dynamic cycles of NCCAs and CCAs occurring within two evolutionary phases (punctuated and stepwise) (Tables 1 and 2, Fig. 1).[24,25] The transition of two phases of somatic cell evolution is linked to stress response, system instability, genome-mediated system replacement coupled with diverse phenotypes and how evolution occurs. Together with the evolutionary mechanism of cancer, it offers explanations to many previous puzzling issues, such as (*i*) why there is elevated genome alteration in the first place. It turns out, in addition to errors from the mitotic process, lower levels of stochastic genome alterations can provide an adaptive advantage in response to stress and/or functional compensation, and they can also initiate further destabilization of the genome and serve as the driving force of cancer evolution as a trade-off;[9,33,47,48] and (*ii*) why it is so common for cancer cells to utilize the genome-level alteration during evolution. The genome serves as the selective entity and platform for gene interaction, which is much more powerful than individual gene mutations. One way cells can acquire such massive genome alterations, is through a phenomenon termed genome chaos,[25,26,49,50] also referred to as chromoplexy and chromothripsis.[37,38] This process, driven by both internal and external stressors, occurs within the punctuated phase and contributes to the diversity necessary for

Table 2. Brief summary of the genome theory of cancer evolution

(*i*) The genome is not just the collection of an organism's DNA. The genome organizes the interactive relationship among genes (of the same chromosome and among different chromosomes). The same or similar genes can form different genomes by re-organizing the genomic topology via karyotypic alteration.[24,42]
(*ii*) Genomic topology defines the genetic network structure. The genome rather than individual genes defines the "system inheritance," while individual genes only provide "parts inheritance."[9,42] The genetic blueprint is about the gene relationships rather than specific genes, as a cell has so many genes that provide sufficient complexity.
(*iii*) Since the genome represents the highest level of genetic organization, its alteration often has a much larger effect than individual gene alteration does. Despite that chromosome changes can impact on hundreds or thousands of genes, an altered karyotype ensures new emergent properties. Rearrangement of the genome can change overall genomic information patterns without generating aberrations in specific cancer genes.
(*iv*) The relationship among gene mutations, epigenetic changes, and genome changes can be illustrated by the multiple level landscape model where local landscape represents gene/epigene status and global landscape represents the status of genome replacement. Fundamentally, the impact of lower levels of alteration (which modify the system) needs to reach to higher-level change to create new systems. Thus the evolutionary mechanism of cancer can be explained by the collection of all molecular mechanisms.[35]
(*v*) Cancer evolution can be described as two phases of evolution[33] (see Table 1). The key for cancer to become successful by overcoming all levels of constraint is to generate heterogeneity and differ from normal cells. This is most effectively accomplished through genome instability-mediated genome re-organization (including genome chaos).[26]

key transitions of cancer.[24] Recently, cancer genome sequencing has forcefully confirmed both the concept of punctuated cancer evolution and the importance of genome chaos.[37–39,51,52]

In order to apply the genome theory to unify the hallmarks of cancer, one needs to understand more about the hallmarks. What are the relationships among these hallmarks, and what is the common basis for these hallmarks? While each hallmark needs extensive molecular description, fundamentally, they all simply represent "difference" between normal and cancer cells/tissues when viewed through the lens of somatic cell evolution.

On the surface, it seems essential to identify key differences specific to cancer so that they can be differentially targeted. However, the fact that so many different genes or even the same genes can contribute or not contribute to these differences or even switch from their main function (depending on genomic and environmental contexts) makes applying them in therapeutics extremely difficult. Increased evidence fully supports our prediction. For example, recent experiments in mouse tumor models have revealed tumor-suppressing effects of genes previously classified as oncogenic.[53] In a previous study, co-expression of CDK4 and a Ras mutant in human epidermal keratinocytes resulted in the abilities to form colonies in soft agar and to develop invasive tumors in mice;[54] however, this co-expression was not found to support unlimited growth in culture. An increased hTERT protein level was observed in the xenograft tumor but not in the cells from culture, supporting that factor(s) from the host mouse resulted in induction of hTERT and supported continuous proliferation, while challenging the basic concept of tumor biology that specific gene combinations are able to immortalize primary human cells.[55] Therefore, even if we could identify and classify the molecular mechanisms underlying these differences for an individual patient, this knowledge would have limited clinical significance, as it would only represent transient probabilities. Within the context of genome instability and tumor cell population dynamics, one would also anticipate conflicting effects (*e.g.* cell death, cell proliferation, no effect, adverse effect) among cells during treatment regimens as a result of targeting specific hallmark characteristics.[56,57]

Therefore, due to evolutionary dynamics, tracing a key feature is challenging as specific features may come and go during evolution, and the initial key factors might or might not play a role in the future. Perhaps the approach of dissecting the system into the simplest terms does not work well in highly dynamic situations such as cancer evolution where genome heterogeneity rules. Multiple level landscape models illustrate this principle well, where there are so many pathways that can achieve fitness, and it is therefore challenging to predict specific pathways.[9,24,58]

Paradoxically, the seemingly complex relationship between hallmarks can be easily unified or understood by using the evolutionary framework of cancer. First, such framework is based on the cancer evolution principle, the most fundamental understanding of cancer. It takes chromosomal instability (CIN) into serious consideration, as the genome carried inheritance (or system inheritance) is a key component for somatic cell evolution.[24,42,47] Despite that CIN has received less attention in the hallmark papers, cancer cell phenotypic variation, tumor heterogeneity, and cancer evolution have been attributed to CIN,[59–62] and this key feature can be linked to all of the hallmarks. We have shown that elevated levels of genome alterations are common in preneoplastic stages both *in vitro* and *in vivo*[63] and highly associated with key stages of cancer stage transition (immortalization, transformation, metastasis, and drug resistance). CIN has been demonstrated to be the result of a wide variety of stresses including oncogene activity, infection and immune response, and temperature and metabolic change.[9,35,49] The hallmarks of cancer themselves provide additional stress to the system,

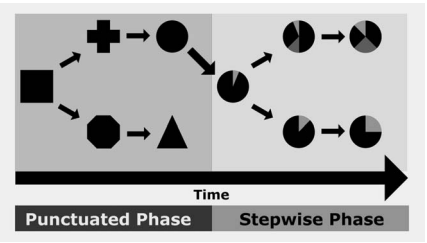

Figure 1. Stochastic model of cancer evolution. Cancer evolution is divided into two distinct evolutionary phases, the punctuated stochastic phase and the stepwise gradual phase. Punctuated phases are marked by extreme heterogeneity and rapid genome changes, represented by genome system changes over time, with each shape representing a unique genome. The punctuated phase is caused by system instability-mediated macro-cellular evolution. Following selection pressure, a unique genome system survives (circle). In contrast to genomes in the punctuated phase, this new genome system in the stepwise phase remains relatively stable over time, although it does acquire low-level change (represented by pie piece change) such as gene mutations, epigenetic alterations, and small traceable genome-level alterations that aide in adaptation. This lower-level genetic diversification refers to micro-cellular changes, which can be classified into clonal expansion and diversification. Thus, the stepwise phase is mainly associated with system stability and micro-cellular evolution. Only one run of the NCCA/CCA cycle is presented.

such as uncontrolled cell growth resulting in tissue and organ stress, suggesting that the hallmarks of cancer also contribute to overall CIN in a stochastic fashion. The consequences of stress-induced CIN, regardless of the different molecular mechanisms that may be sources of stress at a particular time, are genomic variation, breakdown of system constraints and homeostasis, and increased evolutionary potential of the disease (Fig. 2). Therefore, CIN-mediated genome reorganization acts as a major means for cellular heterogeneity and cancer evolution by increasing evolutionary potential and robustness of the disease.[9]

Second, evolution always uses what is available at any given moment rather than waiting for a specific feature. There are many potential features that can be selected, and the selection force constantly changes, as reflected by the high levels of genome alteration. With tumor cell population sizes of 10^9 to 10^{12} cells, and the CIN rate of approximately 10^{-6} per cell, per division, every region of the genome should acquire amplifications and deletions every cell generation. Thus, tumors can rapidly test all possible mutations for fitness benefits, especially when utilizing genome chaos, which allows for higher-level, massive genomic reorganization and new system formation. Fortunately for cancer and unfortunately for patients, NCCAs often exist within cell populations that are suitable for evolutionary selection.

Third, it is highly unlikely that future changes can be predicted based on historical changes when there are many combinations of pathways and genome/environmental interactions that define the biological meaning of gene mutations and pathways. Because of this, genome instability becomes the most important hallmark of cancer, as it can be linked to any of the individual hallmarks and serves as the basis for generating huge numbers of potential packages ready for evolutionary selection, regardless of which hallmark is actually selected from which case or at what stage. While the status of the genome is mentioned as an important characteristic of cancer in light of the hallmarks, attention continues to remain focused on mutations in caretaker genes of the genome that are believed to ultimately drive tumor progression.[5] Since the general causative mechanism of evolution is heritable heterogeneity and the selection process, no single molecular mechanism can explain any cancer until they are unified with the evolutionary mechanisms. Recently, transcriptome dynamics have also been linked to evolutionary potential through genome chaos.[64,65] In addition, models have illustrated how genome dynamics can alter the transcriptome, leading to new evolutionary potential.[65,66] Thus, it is sufficient to use the evolutionary mechanism to understand cancer and use the degree of genome instability to monitor the dynamics of evolution. In contrast, focusing on specific individual molecular mechanisms will not work for highly dynamic cellular populations, where pathway switching, genome system replacement, and new genome system selection are constantly occurring. Obviously, a holistic and/or ecological approach (such as measuring the NCCA/CCA index or measuring the diversity itself) that focuses on monitoring overall levels of population system stability/instability, genomic heterogeneity, and evolutionary potential are more useful than monitoring just one or a few specific features when there are high levels of dynamics involved. Again, such explanation can be illustrated using adaptive multiple level landscape models.[31,58] Further, the hallmarks of cancer have recently been categorized as proliferation and survival phenotypes.[67]

Based on the current knowledge of the genome and the hallmarks of cancer, the gap between the conceptual understanding and clinical applicability of these biological principles and molecular pathways can be remedied by the new approach of the genome theory. The first step is establishing the prevailing principle that unifies the hallmarks of cancer, which the genome theory identifies as the stochastic genome variation of macro-cellular evolution within the punctuated phase. While each molecular mechanism can serve as a system stress and other factors such as genetic and epigenetic variations as well as environmental influences play a role in cancer progression, the commonality between them is the presence of system-level dynamics, reflected by stochastic genome variations, which ultimately create new genetic networks.[1] Furthermore, the stochastic model of evolution helps to explain the clinically observed phenomenon that each tumor is different when comparing cancer cell samples.[25] While cancer cells may share similar phenotypes, the genetic

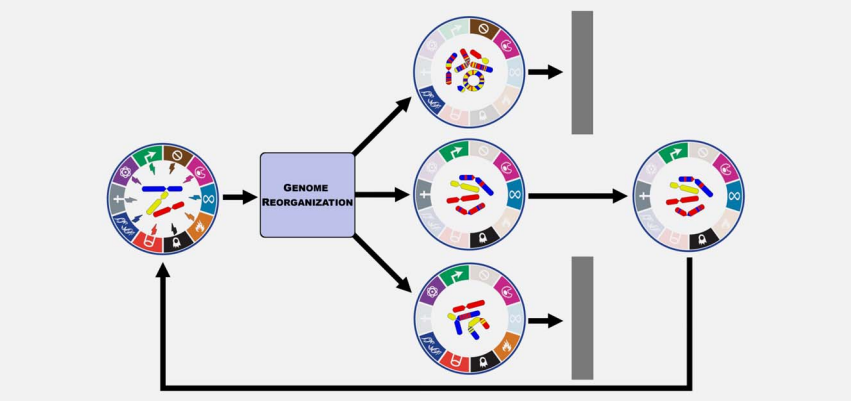

Figure 2. Diagram depicting the impact of stress-induced chromosomal instability in the context of genome-mediated cancer evolution. Different chromosomes of the genome are designated by color (blue, yellow, red). The hallmarks of cancer (shown surrounding the chromosomes, hallmark symbols derived from Ref. 5) can serve as sources of stress to the genome as represented by lightning bolts, resulting in rapid, stochastic genome fragmentation and genome topology reorganization. The consequences of this process are increased karyotypic heterogeneity and evolutionary potential, as shown by numerical aberrations (*e.g.* aneuploidy) and/or structural aberrations (*e.g.* translocations). Breakdown of system constraints and alteration of genome topology result in phenotypic variance, as represented by different highlighted hallmark combinations for each genome system. Stable karyotypes that survive this process are selected for clonal expansion, and this process cycles upon future internal/external crisis events. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

route taken to get there is likely to be unique and driven by evolutionary opportunity, resulting in dissimilar tumors at the genetic level.[68] This model also sheds light on the failure to translate gene-directed linear processes derived from clonal model system utilization and experimentation to the clinical reality of overwhelming primary tumor heterogeneity. Thus, the genome theory can provide direction as a comprehensive approach that accounts for the multiple mechanisms involved in the hallmarks of cancer by equating the evolutionary mechanism to the sum of all of the individual mechanisms. This relationship is consistent with the fact that each molecular mechanism can contribute to cancer yet there is not one presiding mechanism found across all cases. Therefore, pathways must be considered collectively within the context of the entire system. The evolutionary mechanism of cancer also accounts for the diverse genetic and epigenetic alterations and acknowledges that cancer, with the help of these hallmark principles, is ultimately achieved through multiple cycles of macro-cellular evolution that are stochastically determined.[25]

Clinical Implications

It is now clear that the effort to link individual gene mutations to the hallmarks offers limited clinical value, as in contrast to the data from basic research efforts based on linear models, there is no common molecular basis leading to cancer evolution in general. By analyzing each tumor, we can illustrate only end stage dominant mechanisms. The real challenge lies in predicting tumor response and progression, similar to how we can comprehensively analyze an historical event, but it is difficult to translate that information towards making predictions for the future. Examples of the shortcomings of applying linear model logic in treatment have extended beyond direct cancer gene targeting. For example, recent efforts have focused on targeting stromal cells and manipulating the tumor microenvironment (*e.g.* antiangiogenesis therapy). The rationale behind this approach is to stop tumor blood supply, thus killing the tumor. This approach has shown high promise in animal models; however, it has been much less successful in the clinic, as rapid onset of resistance occurs despite the targeting of endothelial cells rather than cancer cells.[56,69] Even more troublesome, integrin inhibitors can paradoxically promote tumor angiogenesis and enhance tumor growth, despite that integrin inhibitors block integrin action and the angiogenesis process in tumor models.[56,70]

Another recent approach involves focusing on the epigenetic level, as DNA methylation and histone modification have a profound effect on the epigenetic regulation of gene expression, and these processes could serve as therapeutic targets. Unfortunately, early attempts of using DNA methyltransferase inhibitors have generated mixed results from different model systems. Strong demethylation was also linked to genome instability.[56,71] Similarly, histone deacetylase

inhibitors have been applied to cancer treatments, and the results have not been that promising as their efficacy is limited and, in addition, these inhibitors could also promote tumor growth.[56,72] These mixed findings, from an evolutionary perspective, can be easily clarified. According to the evolutionary mechanism of cancer, significant stress (direct or indirect) will result in increased system dynamics, generating genome level heterogeneity necessary for cancer cell evolution. This increased diversity gives the disease a chance to survive when the evolutionary landscape changes as a result of clinical intervention, for example. Therefore, the outcomes are highly unpredictable. While specific molecular targeting may be an ideal strategy in a stable system, it is not a reasonable approach for an unstable, evolving system that is characterized by an unlimited number of potential pathways and dynamic changes to the transcriptome as a result of genome system alteration.[9,64,65,73] Therefore, to achieve a clinically relevant understanding, we must utilize approaches that account for inter and intra-tumoral heterogeneity in cancer.[74]

Such unpredictability can be most obvious when dealing with treatment. As previously demonstrated, high doses of chemotherapeutics intended for high rates of tumor cell death that are commonly prescribed to patients can trigger chaotic genome formation and rapid changes of genome systems, which may lead to rapid emergence of an aggressive, drug-resistant tumor subpopulation.[24–26,57,73] Thus, the genome theory calls into question the current standard protocols of chemotherapy, as drug intervention could paradoxically promote cancer evolution when applied in the wrong phase.[75,76] Therapeutic strategies should include the aim to reduce system stress to avoid triggering fast cancer evolution.

Conclusions and Future Perspective

The intent of this analysis is not to downplay the initial importance of studying the molecular linkage between hallmarks of cancer and molecular principles, but rather to place the utility of these hallmarks in the true perspective of cancer evolution, encourage researchers to realize the limitations of this concept, and call for new frameworks.

This new framework, the evolutionary mechanism of cancer, not only can integrate diverse components such as hallmarks into the dynamic process of cancer evolution, but also unify and even simplify our understanding.[31] The evolutionary mechanism of cancer consists of three key components: (*i*) system stress (biological process itself including metabolic dynamics, aging, bio-system errors, environmental challenges, cellular adaptive processes), (*ii*) population diversity (at phenotype level, the hallmarks, and their dynamics; at genotype level, there are multiple levels of genetic and non-genetic inheritance which can contribute to the hallmarks in a less predictable fashion), and (*iii*) genome-mediated macro-cellular evolution (since the genome is the platform that organizes emergent properties and passes system inheritance, genome-level alteration is the driving force to achieve the evolution of new systems rather than individual features/hallmarks, despite that lower-level genetic changes can influence genome-level changes).[25,35,36]

Significantly, such simple framework can solve many well-known paradoxes in the field. First, various types of genomic and non-genetic variation are not just "genomic errors," but these serve the important biological functions of stress response and short-term adaptation. As a trade-off, they also contribute to the increased potential for cancer in the long-term.[47,48]

Second, most individual runs of cancer evolution are not successful within the human body due to the multiple levels of system constraint and the low probability of the "perfect storm" required by cancer to become clinically significant.[1] Breakdown of system constraint (for example through the aging process or prolonged high stress cellular conditions) is the major contributing factor for successful cancer evolution. Since somatic cell evolution relies on inheritance, and the genome defines the system inheritance,[24] genome alterations become the common drivers for somatic evolution. To reduce the likelihood of cancer evolution, stabilizing the normal genome is the key. This idea is clearly supported by the function of sex, as the constraint of genome integrity established during sexual reproduction ensures species identity.[77–79] When the system is unstable, generation of highly diverse cell populations with seemingly endless potential is possible, increasing the probability to form the perfect storm. As a result, the power of prediction using cancer hallmarks becomes very limited.

Third, there is inconsistency between "part characterization" (such as studying an individual hallmark and its molecular elements) and evolutionary selection based on the entire system (such as estimating the overall heterogeneity-defined evolutionary potential).[9] We have referred such inconsistency as a knowledge gap.[31] Interestingly, common molecular approaches in fact hugely contribute to this gap. In addition to the limitation of the reductionist approach,[30] many molecular understandings are derived from experimental systems that are based on some key assumptions. For example, some assume that cancer progression is like the normal development process where stepwise molecular events can be identified. Others assume identified molecular mechanisms based on homogeneous cell populations and average profiles can apply to patients in the clinic. However, the fact that cancer is typically highly heterogeneous (especially at the genome level, which is drastically different from the development process or normal physiological conditions),[32,33,40,80,81] and that cancer evolution is a stochastic process[35,36] makes some molecular information less significant in terms of understanding the disease. In a sense, most of the molecular mechanisms we know perhaps are all correct (in the sense of representing a possibility under certain conditions) but not very useful in the clinic (due to the low penetration in the patient population and evolutionary dynamics complexity).

Fourth, the movement to increase molecular resolution and collecting more and more high-resolution data needs to be

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questioned. By discussing the limitation of focusing on individual hallmarks of cancer, we are actively promoting that lower-level genetic alteration is less effective for cancer evolution where punctuated evolution is achieved by genome replacement with much less predictability. Thus this high level of complexity can only be appropriately understood by measuring genome heterogeneity, overall stability/instability status and evolutionary potential of the disease to develop estimates of treatment response and disease progression.[33,34,47]

With this new understanding, we need to divert our efforts from creating linkages that do not translate well to the clinic and instead move forward to studying cancer as a macro-cellular evolutionary process. This would include accounting for system constraint, population behavior, and overall tumor management rather than aggressively targeting and eliminating cancer cells. Experimental trials applying adaptive therapy, where treatment dosage is regulated in order to maintain a stable tumor burden, have shown promise over maximum tolerated dose regimens.[82,83] In order to take the next step in improving cancer diagnosis and treatment, focus must shift toward the maintenance of disease stability as many attempts to eliminate cancer have fallen short while perhaps inadvertently promoted further cancer macrocellular evolution. To achieve this goal, clearly, new strategies beyond what the hallmarks of cancer have offered are urgently needed.

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APPENDIX F: HORNE SD, YE CJ, ET AL. (2015). "CHROMOSOMAL INSTABILITY (CIN) IN CANCER." ELS: 1-9

Chromosomal Instability (CIN) in Cancer

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Advanced article

Article Contents

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Chromosomal instability (CIN) represents a common feature in the majority of cancers. Despite that the search for specific molecular mechanisms linked to the causation or consequences of cancer has become very popular in cancer research, there is no general conceptual framework that unifies the observed diverse molecular findings. By applying the genome theory of cancer evolution, we briefly define and clarify CIN, synthesise its importance in macro-cellular evolutionary selection, unify diverse molecular mechanisms under the evolutionary mechanism of cancer and discuss its potential implications. Understanding the relationship of stress, CIN and genome-mediated cancer evolution offers clarity and direction to researchers, and monitoring CIN within an evolutionary context can provide valuable clinical information for determining treatment administration and patient prognosis.

Introduction

Chromosomal instability (CIN) in cancer has recently become a hot research topic (Schmutte, 2005; Heng *et al*., 2006, 2011a, 2013a, b; Ye *et al*., 2007; Pfau and Amon, 2012; Burrell *et al*., 2013). Given the fact that chromosomal aberrations overwhelmingly exist in the majority of cancer types, this should not surprise anyone. However, just a few years back, CIN research has been rather limited. The research scope was mainly focused on chromosome instability syndromes (such as Bloom syndrome and ataxia telangiectasia) and associated DNA damage/repair pathways, and chromosomal instability has been considered the direct consequence of specific cancer gene mutations. Influenced by this thinking, the contribution of CIN in cancer evolution has been largely ignored, as researchers were most interested in characterising gene mutations that could be directly linked to CIN. As for how CIN could stochastically promote cancer evolution, and especially how general stress can elevate CIN and contribute to sporadic cancer, little was known.

In recent years, the situation has drastically changed. The importance of the karyotype was realised with the discovery of system inheritance. The chromosome is not just the vehicle of genes, but rather the genetic organiser that determines the genetic network structure by maintaining the physical interactions among genes within the three-dimensional nucleus. More specifically, the role of the genome topology is to provide the framework under which the genetic network is governed. In other words, the karyotype defines the system inheritance or blueprint of an organism, whereas individual genes/epigenes only represent the 'parts inheritance', as the same genes can be reorganised to form different systems. As a result, any significant chromosomal alteration can change the blueprint by forming a new genome system (Heng, 2009; Heng *et al*., 2011b). Such realisation underscores the ultimate importance of stochastic chromosomal aberrations and calls for no longer considering these seemingly random chromosome aberrations as insignificant 'noise'. As CIN is the engine that produces all types of chromosomal aberrations, which serve as the basis for genome heterogeneity in somatic cell evolution, it is obvious that understanding CIN is the key to understanding cancer. In addition, cancer sequencing projects have revealed overwhelming degrees of genome heterogeneity, challenging the framework of the somatic gene mutation theory in cancer research that has been focused on individual gene characterisation and ignored the utmost important contribution of the genome alterations observed in the vast majority of cancers (Heng, 2007; Navin *et al*., 2011; Stephens *et al*., 2011; Gerlinger *et al*., 2012; Baca *et* al., 2013). Despite this exciting progress, there are many issues that must be discussed in this rapidly moving field. In this article, we briefly define and classify CIN in the context of cancer evolution.

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What Is CIN, and Why Is It the Key Driver of Cancer?

CIN can be defined as an increased rate of gross chromosomal alterations (or altered karyotypes) within a given cell population. These alterations range from numerical aberrations (e.g. aneuploidy and polyploidy), structural aberrations (e.g. translocations, deletions and many more unclassified types) (**Table 1**) or a combination of both. Loss of heterozygosity (LOH) and copy number variation (CNV) could also be included when these involve large sequence sizes.

To understand why CIN is so commonly detected in most cancers, one needs to appreciate the pattern of cancer evolution and how CIN serves as the common driving force in cancer evolution. First, as illustrated by an *in vitro* immortalisation model, the two phases of somatic cell evolution (punctuated and stepwise) were initially observed during the immortalisation process (Heng *et al.*, 2006). Such cyclical phase transition was also detected from cellular transformation, metastasis and drug resistance through

Table 1 Examples of karyotypic abnormalities

Category	Type	References
Structural	Translocation	ISCN 2013: An International System for Human Cytogenetic Nomenclature, eds Shaffer LG *et al*. Basel: Karger
	Deletion	
	Insertion	
	Inversion	
	Duplication	
	Triplication	
	Quadruplications	
	Ring chromosomes	
	Fission	
	Fragile sites	
	Dicentric chromosomes	
	Derivative chromosomes	
	Telomeric associations	
	Premature chromosome condensation (or pulverisation)	Johnson RT & Rao PN. (1970). *Nature* **226**(5247): 717–722
	Micronuclei	Fenech M *et al*. (2011). *Mutagenesis* **26**(1): 125–132
	Multipolar mitosis	Gisselsson D. (2001). *Atlas Genet Cytogenet Oncol Haematol* **5**(3): 236–243
	Chromosome bridge	Gisselsson D. (2001). *Atlas Genet Cytogenet Oncol Haematol* **5**(3): 236–243
Numerical	Aneuploidy	ISCN 2013: An International System for Human Cytogenetic Nomenclature, eds Shaffer LG *et al*. Basel: Karger
	Polyploidy	
	Endopolyploidy	
Non-traditional/ Newly identified	Free chromatin	Heng HQ *et al*. (1988). *Mutat Res* **199**(1): 199–205. Heng HH *et al*. (1992). *Proc Natl Acad Sci U S A* **89**(20): 9509–9513. Heng *et al*. (2013b)
	Defective mitotic figures	Heng HQ *et al*. (1988). *Mutat Res* **199**(1): 199–205. Haaf T & Schmid M. (1989). *Chromosoma* **98**(2): 93–98. Smith L *et al*. (2001). *Proc Natl Acad Sci U S A* **98**(23): 13300–13305. Heng HH *et al*. (2004). *Cell Chromosome* **3**(1): 1
	Sticky chromosomes	Heng *et al*. (2013b)
	Unit fibres	Heng HQ *et al*. (1988). *Nucleus* **30**: 2–9
	Chromosome fragmentation (C-Frag)	Stevens *et al.* (2007)
	Genome chaos/karyotype chaos	Heng *et al*. (2006) Duesberg P. (2007). *Sci Am* **29**(8): 783–794. Heng (2007) Liu *et al*. (2014)
	Karyoplast budding	Walen KH. (2005). *Cell Biol Int* **29**(12): 1057–1065
Unclassified	Nuclei with small holes	Heng *et al.* (2013b)
	Giant nuclei	Heng *et al.* (2013b)
	Irregular interphase morphology	Heng *et al.* (2013b)

chaotic genome formation (Heng *et al.*, 2013a; Horne *et al.*, 2015b). Recently, various cancer genome sequencing studies have confirmed the punctuated phase (Wang *et al.*, 2014; Sottoriva *et al.*, 2015). Importantly, the punctuated phase is linked to genome replacement mediated macro-cellular evolution, whereas the stepwise phase is linked to gene/epigene mutation-mediated micro-cellular evolution (Heng *et al.*, 2006; Klein, 2013; Heng, 2015). The fact is, without triggering CIN, even some powerful oncogenes will fail to initiate cancer.

Second, CIN has been directly linked to various stress conditions (genetic and non-genetic alike) (Stevens *et al.*, 2011a) as well as tumourigenicity and aggressiveness (Ye *et al.*, 2009). CIN-induced genome dynamics reflect as an elevated transcriptome (Pavelka *et al.*, 2010; Stevens *et al.*, 2014). The level of transcriptome dynamics was also associated with karyotype stability. Interestingly, during phases of punctuated evolution where karyotypes are highly unstable, transcriptomes observed were also unstable, suggesting the linkage between CIN and functional heterogeneity. It is important to note that each run of an immortalisation model resulted in different end products (i.e. karyotypes and transcriptomes), suggesting that each run of cancer evolution is different, without common drivers (Stevens *et al.*, 2014). Thus, high levels of CIN can stochastically provide ample opportunities for cancer evolution.

Third, increased CIN is associated with the increased success of outliers (Abdallah *et al.*, 2013). When CIN is high, cellular features become highly heterogeneous within the cell population, including individual cell growth, transcriptome and cell death (Abdallah *et al.*, submitted). CIN, especially under highly stressful conditions, can significantly increase the odds and speed of outlier success. This has huge implications for drug resistance.

Considering these findings, the true value of CIN is to increase the evolutionary potential for precancerous and cancer cells. As the key for cancer evolutionary success is being different from normal cells (for effective selection), CIN becomes the common driver for cancer evolution, and especially for macro-cellular evolution. This conclusion fits well with many important observations, such as the strong correlations between CIN levels and tumourigenicity (Ye *et al.*, 2009), tumour grade, metastasis, drug resistance and poor prognosis (Sheffer *et al.*, 2009; Heng *et al.*, 2013a).

Whether or not CIN is a good or bad index of cancer formation has been a source of puzzlement. On one hand, CIN is present in nearly all cancers and is commonly observed during key transitional events (e.g. immortalisation, metastasis and drug resistance), supporting its important contribution to cancer. On the other hand, CIN in cancer has been associated with poor proliferation in experimental models (Pfau and Amon, 2012), which may suggest that CIN could also slow down cancer formation and even could be a therapeutic avenue based on its association with slower tumour cell growth. We have previously discussed the relationship between CIN and poor proliferation using the NCCA/CCA cycle (Ye *et al.*, 2007). It is clear that less CIN is often associated with a growth advantage but with a survival disadvantage. In other words, genome instability ensures survival by providing a wide variety of genomes, even though not all will be fit for a particular environment at a particular time (resulting in slower growth as a trade-off). The key here is generating survivors with different genomes and maximising the odds (or diversity) for cancer cell survival (gene mutations alone are not enough). This sharply contrasts with the relationship between genome stability and rapid proliferation that occurs after macro-cellular evolutionary selection. The importance of CIN also reinforces the idea that cancer evolution is not just about proliferation but the emergence of new genome-defined cellular systems (Heng, 2015).

What Are the Common Types of Chromosomal Variations Indicating CIN, and Why It Is Challenging to Monitor CIN?

CIN can be classified as structural and/or numerical CIN (Heng *et al.*, 2006; Bayani *et al.*, 2007). Numerical CIN is determined by gain or loss of whole chromosomes or fractions of chromosomes, whereas structural CIN is determined by structural non-clonal chromosome aberrations. Even though most researchers are familiar with classical chromosomal aberrations such as translocations and aneuploidy, the list of structural aberrations reflecting CIN is extensive and highly diverse. Following the realisation of the importance of NCCAs (Heng *et al.*, 2006), the list of NCCAs has quickly expanded. It includes familiar aberrations such as simple translocations, complex duplications, deletions, double-minute chromosomes, homogeneously staining regions, multicentric chromosomes, ring chromosomes, lagging chromosomes, small supernumerary marker chromosomes and multi-radial chromosomes (Ye *et al.*, 2007; Stevens *et al.*, 2007, 2011b). In addition, there are recently discovered, often ignored and unclassified aberrations including defective mitotic figures, chromosome fragmentation, free chromatin, sticky chromosomes, micronuclei, genome chaos (which includes recently characterised subtypes such as chromothripsis and chromoplexy), giant nuclei and other abnormal nuclear morphologies (Stephens *et al.*, 2011; Baca *et al.*, 2013; Heng *et al.*, 2013a, b) (**Table 1**; **Figure 1**).

Many NCCAs have been continually ignored because of their un-clonal nature. One major conceptual error has been using CCAs to monitor CIN (as NCCAs were previously considered insignificant genetic noise). In fact, quite the opposite, CCAs represent stability rather than instability (Heng *et al.*, 2013a). Furthermore, the unfamiliarity of many structural NCCAs has further lowered estimations of CIN in monitoring cancer cell populations. More importantly, most NCCAs are undetectable by averaging molecular methods, and single cell analysis is required. This is the reason why classical cytogenetic analyses are needed to study CIN. Finally, to correctly monitor CIN, a large number of cells are necessary. For our SKY analyses, for example, 50–100 individual cells are needed. This poses an additional challenge for using single-cell sequencing to study CIN, as the costs could be very high compared with cytogenetic methods. Unfortunately, many current molecular cytogenetic methods such as array CGH are not suitable to profile CIN because of their nature of profiling the average of the cell population.

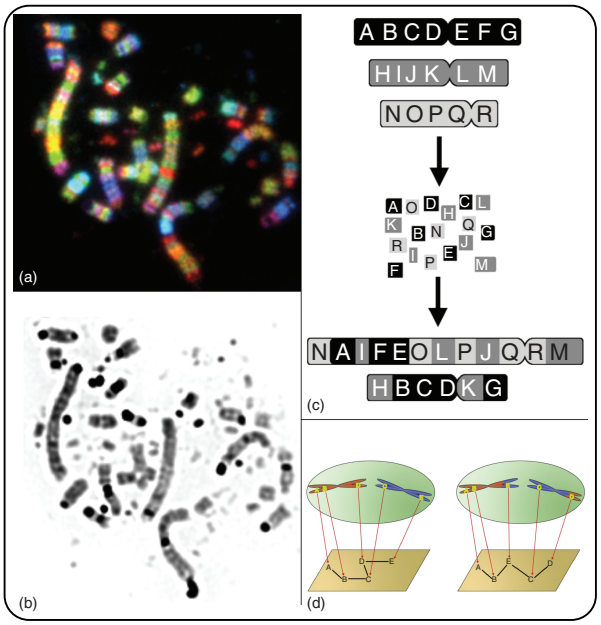

Figure 1 The phenomenon of genome chaos generating new genetic systems. When CIN becomes extremely elevated under high stress, formation of chaotic genomes will be induced. The drastically altered genome will result in a new system with a newly formed network structure. (a) Spectral karyotype (SKY) image of genome chaos where massive translocation events are detected within a chaotic genome following drug treatment. These newly formed giant chromosomes are possibly derived from complex chromosomal fusion following chromosome fragmentation, with each colour representing their chromosomal origin. (b) The reverse DAPI image of the same mitotic figure in (a). (c) Schematic demonstrating how various forms of genome chaos may occur. Normal chromosomes are shown at the top, with each letter within the chromosomes representing a distinct region. Following exposure to sufficient degrees of various stressors, the genome undergoes partial fragmentation. Following fragmentation, regions are recombined and rejoined, resulting in the genome chaos demonstrated at the bottom. Newly formed chimeric chromosomes can be a mixture of various chromosomal origins, or occasionally from a single chromosome. (d) Changes in genome topology alter genetic network structure. For simplicity, two chromosomes are drawn within the nucleus, representing the genome. Genes are designated A, B, C, D and E within the chromosomes. When a translocation occurs, the genome topology is altered, affecting the physical relationship between chromatin domains and changing the overall genetic network structure. As a result, the genetic network changes (indicated by the altered relationship among proteins A, B, C, D and E). Thus, drastically altered genomes (products of genome chaos) represent new genome systems, and understanding this process provides insight into macro-cellular cancer evolution. Reproduced with permission from Heng *et al.*, 2011a,b © Elsevier.

What is the Mechanism – The Molecular and Evolutionary Causes and Consequences – of CIN?

Gene mutations/chromosomal machinery aberrations and CIN

CIN research is frequently aimed at identifying key genetic factors responsible for the maintenance of genetic integrity. On the basis of the gene centric concept of genetics, initial efforts have been focused on identifying gene mutations and molecular pathways that lead to replication and DNA repair errors. Well-known examples include genome instability syndromes with characterised gene mutations, such as mutations in the RecQ helicases in Bloom, Werner and Rothmund-Thomson syndromes (van Brabant *et al.*, 2000), mutations in genes involved in DNA double-strand break repair in Nijmegen breakage syndrome (D'Amours and Jackson, 2002) and gene mutations that are associated with hereditary cancers such as BRCA1 or BRCA2 (in early onset of breast cancer) and in some familial cancer types such as p53 mutation in Li-Fraumeni syndrome. In addition to these rare cancers, DNA replication errors are extensively studied for the understanding of CIN and cancer. Many molecular associations have been made to CIN, including genes involved in DNA repair, cell cycle regulation and cell death pathways (**Table 2**).

Table 2 Classification of major contributors of chromosomal instability

Type/definition	Categories (genetic factor examples)	References	Challenges/limitations
Type I mechanisms Molecular factors hold a direct causative relationship with CIN. Includes factors that are directly linked to the maintenance of genome integrity	*Chromosome integrity* Chromosome condensation/decondensation Chromosome segregation (BUB1) Chromosome fragment transfer Cytokinesis Telomere shortening Centrosome instability/duplication (TP53, RB1, APC) Inhibition of retrotransposons Incomplete mitotic cell death Chromosome repair Non-homologous end joining Homologous end joining 3D chromatin domain interaction Fragile sites Highly transcribed DNA sequences Chromosome breakage-fusion-bridge cycles (TP53, MDM2) *DNA integrity* DNA replication Cell cycle checkpoint pathways/kinases defects (TP53, ATM, CHK2, MAD2, BUB1, RB1) *DNA repair* Mismatch repair (MLH1, MSH2, MSH6, PMS2) Nucleotide excision repair (XPA-XPG genes, XPV) Base excision repair (MYH) *DNA methylation status maintenance* Hypermethylation Hypomethylation Histone modification	Schmutte C. (2005). *eLS*. John Wiley & Sons, Ltd: Chichester Gisselsson D. (2001). *Atlas Genet Cytogenet Oncol Haematol* **5**(3): 236-243 Gisselsson D. (2001). *Atlas Genet Cytogenet Oncol Haematol* **5**(3): 236–243 Gisselsson D. (2001). *Atlas Genet Cytogenet Oncol Haematol* **5**(3): 236–243 Schmutte C. (2005). *eLS*. John Wiley & Sons, Ltd: Chichester Perera S & Bapat B. (2007). *Atlas Cytogenet Oncol Haematol* **11**(2): 155–164	Low clinical prediction value; having a type I CIN mutation does not make cancer a certainty. Rare, do not explain sporadic cancers
Type II mechanisms General system dynamics stress response as the result of various factors (less molecular specificity) that do not display a direct causative relationship with CIN. Linked to a CIN phenotype under certain conditions. More common than type I mechanisms in cancers. Responses provide evolutionary adaptation advantages, such as increased fuzzy inheritance and overall disease robustness	*Physiological stresses* Ageing Metabolism Hormones Inflammation Wound healing *Environmental stresses* Therapeutics Infection Toxins Pollution *Responses with adaptive and survival advantages* Immunological diversification Liver cell adaptation Genome chaos under crisis		High complexity and dynamics make it difficult to determine specific contributions to CIN and observe direct causation. Stochasticity negatively impacts clinical prediction value of specific mechanisms and requires a holistic, evolutionary understanding

With the increased appreciation of chromosomal aberrations in cancer, the research focus on CIN has begun to shift towards chromosome machinery itself, and gene mutation leading to the generation of chromosomal abnormalities is under investigation. It is understood that defects in key genetic pathways result in chromosomal aberrations as a consequence or by-product, which contribute to the cancer formation. Examples include the linkage of the BUB1B mutation to mosaic variegated aneuploidy and predisposition to various types of cancer (Hanks *et al.*, 2004). Multiple causes of abnormal chromosomal machinery have been identified including mitotic checkpoint defects, cohesion loss and merotelic kinetochore attachment (Janssen and Medema, 2013).

In addition, other stages of the chromosomal cycle including chromosome condensation defects are also involved. It is thus easy to predict that many more gene mutations will be identified that are responsible for chromosomal integrity (Heng *et al.*, 2013a).

However, explanation power based on direct linkage between CIN and DNA/chromosomal machineries is limited, especially when applying this knowledge to explain the majority of sporadic cancer cases. For instance, germline mutations of BUB1B are rare in human tumours, and individuals with DNA repair gene mutations can be cancer-free even at older ages. Furthermore, there is a paradoxical effect of CIN on cancer, as aneuploidy can function either to inhibit or to promote tumourigenicity (Weaver and Cleveland, 2007). Obviously, there seems to be another unknown layer of factors, which is more important for CIN.

General stress, adaptation and the evolutionary mechanism of CIN

The above-mentioned reasoning has led to a new effort to search for the common mechanism of CIN in the majority of sporadic cancers, which might not be directly linked to common driver mutations for a small subgroup of patients. On the basis of the facts that, the CIN phenotype can be linked to a large number of genes in yeast (Kolodner *et al.*, 2002), and various stresses (caused by genetic or non-genetic factors) can be linked to CIN (Heng *et al.*, 2006; Stevens *et al.*, 2011a), the model of stress-CIN-adaptation was proposed, which classifies CIN into two types (Heng *et al.*, 2013a) (**Table 2**). Type I includes mechanisms that are directly linked to the maintenance of genome integrity throughout the DNA and chromosomal cycle, including the chromosomal machinery, checkpoints, and repair systems. Type I mechanisms are often associated with CIN syndromes and are straightforward because of the direct relationship between the identified factor(s) and CIN. However, mutations in type I genes are rare, and they do not explain sporadic cancers. Type II CIN mechanisms are those that do not have a direct molecular causative explanation. However, they are clearly linked to a CIN phenotype under certain conditions. It was reasoned that type II mechanisms are more common than type I mechanisms because of the infrequency of type I mechanisms in sporadic cancers. Type II mechanisms are frequently linked to non-genetic factors such as the micro-environment and physiological processes (e.g. ageing, hormones, inflammation and metabolic status). This understanding has integrated environmental impact into the CIN-cancer framework. To understand the general mechanism of type II CIN, one needs to consider the homeostasis of the entire system. Each individual defect to the whole system (genetic or non-genetic) can be considered a stress (**Figure 2**), and this understanding links both types of CIN. Establishing this link and recognising the importance of type II CIN in fact exposes the limitations of type I CIN in cancer.

Another key is to understand the potential benefit of CIN at the cellular level (depending on the context, any change could be good or bad). Increased cellular heterogeneity as a result of CIN could provide the robustness necessary to perform complex functions and/or survive against environmental stress. Thus, as we have recently discussed, CIN plays a key role in an evolutionary trade-off (Horne *et al.*, 2014). Given the vast karyotypic heterogeneity observed in normal, healthy tissues coupled with stress-induced CIN (from a wide variety of internal and external stresses), CIN serves as an adaptive response necessary for both performing complex cellular and organ functions (e.g. liver detoxification, as the healthy liver displays polyploidy) as well as withstanding the stress that comes as a result of these functions. However, the trade-off is that stress-induced CIN can also lead to the onset of common diseases including cancer.

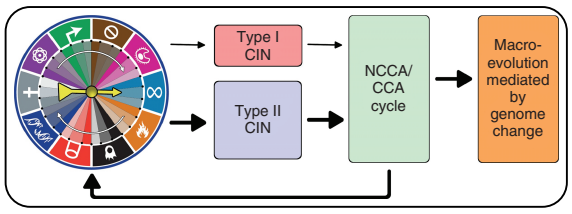

Figure 2 Diagram illustrating the relationship among stress, diverse individual molecular mechanisms of cancer, CIN, and stochastic genome change-mediated cancer evolution. The hallmarks of cancer (adapted from Hanahan and Weinberg, 2011) were used to represent different pathways linked to cancer. Stress is the motor that turns the pathway wheel. Selection of a given pathway as a mechanism of cancer progression is represented by the arrow which selects a pathway based on probability. Individual pathways can directly compromise genome integrity (type I) or indirectly jeopardise genome integrity through general stress (type II). Both types I and II CIN are linked to elevated NCCA frequency. Stress-induced CIN is the key generator of evolutionary potential leading to macro-cellular evolution. Reproduced with permission from Heng *et al.*, 2013a © Springer Science+Business Media.

Departing from a popular stance on CIN, which is that CIN is the consequence (or by-product) of gene-level deregulation, we now understand that CIN provides necessary heterogeneity for both normal tissue and for disease, and it can result from gene mutation and other genetic/non-genetic stresses. As CIN creates a high level of robustness for cancer, making it formidable against treatment, immunological attack, competition with healthy tissues and so on, CIN is the key driver of cancer and the most important player in the equation of stress-adaptation-genome replacement-cancer evolution. Now it is clear that the previously unknown layer of complexity above CIN is the evolutionary platform, as the power of CIN has to be within the context of cancer evolution. For a specific well-defined or controlled case, whether elevated CIN is good or not depends on the biological context, as illustrated by instances where CIN is associated with lower cellular proliferation rates. As elevated CIN is also associated with cell death, some researchers have called for targeting CIN as a therapeutic strategy (Roschke and Kirsch, 2010). However, in general, elevated CIN will favour evolutionary selection in real cancer cases (as opposed to in more linear animal models). Despite increased cell death, elevated CIN will favour the formation and success of outliers, some of which could result in rapid macro-cellular cancer evolution (Abdallah *et al*., 2013; Liu *et al*., 2014; Heng, 2015).

Fuzzy inheritance and CIN

The realisation that CIN plays an important role in cellular adaptation also leads to a new concept; as CIN is not simply an erroneous response to stresses, and there might be an internal mechanism for creating and/or maintaining system heterogeneity. Mutation rates are too low to fulfil the goal of generating so much variation under stress. Using single-cell analysis on cell populations with known degrees of CIN, karyotype inheritances were carefully observed and compared, and the underlying mechanism was termed 'fuzzy inheritance' (Heng, 2015, Heng *et al*., 2015; Horne *et al*., 2015b; Abdallah *et al*., submitted). In normal tissue, there is a lower baseline of NCCAs, where alteration is often minimal with limited chromosome stochasticity; during high-dose treatment-induced genome chaos, however, the fuzziness of a cell population could be reached to its maximum level, where daughter cells no longer display the same karyotype of the mother cell. It is suggested that genome chaos represents a drastic survival strategy for cancer cells, and the high level of fuzzy inheritance plays an essential role for cancer cell survival. This hypothesis states that low levels of fuzzy inheritance are useful for micro-adaptation, whereas high levels of fuzzy inheritance are key for macro-cellular evolution. Even though CIN can be erroneously generated, many instances are not the result of error but are generated by this mechanism to produce necessary diversity! Further examination of this hypothesis is now underway.

Together, the above-mentioned analysis of CIN addresses many important and puzzling questions (Janssen and Medema, 2013). Is CIN an indicator of tumourigenesis? Absolutely. Does CIN mainly provide an enhancing effect to promote transformation? CIN does not just promote transformation, but rather has a determining role. Is CIN a mere consequence of tumour formation? Despite that CIN also reflects tumour status, it is the driver of cancer.

Potential Implications of CIN in Cancer Research and Treatment

As no individual molecular mechanism serves as a clear general mechanism for CIN, focusing on CIN within an evolutionary framework rather than dissecting all potentially involved processes makes sense for both cancer research and its implications. Further, when high levels of CIN are involved, the contributions of driving genes or pathways will not be fixed (and will likely conflict within the tumour cell population) owing to rapid system change and heterogeneity. We have previously introduced the evolutionary mechanism of cancer (EMC) to account for this diversity, which is equal to the sum of all individual molecular mechanisms. The EMC can be detailed in three steps: (1) stress-induced genome system instability; (2) this instability results in genetic heterogeneity at multiple levels, providing necessary diversity for selection and (3) somatic cell evolution, importantly macro-cellular evolution, where system replacement results in breakdown of system constraints (e.g. tissue architecture and immune system). This concept generalises all involved genetic and non-genetic factors, as long as they provide sufficient stress to significantly destabilise the system. Further, as CIN unifies the wide variety of dynamic pathways (and thus unifies the hallmarks of cancer), we have previously argued that CIN represents the key hallmark of cancer (Horne *et al.*, 2015c). From this holistic concept, it is important to shift our research/clinical focus onto a higher level (i.e. genome level) when dealing with CIN, as a wide variety of molecular factors are involved with varied and dynamic contributions. According to the multiple level landscape model of cancer, in order to reach the macro-cellular phase of cancer evolution, genome replacement is necessary (Heng *et al*., 2011a, 2013a; Huang, 2013).

It is important to note that, as CIN impacts dynamics at multiple genetic levels (genomic, transcriptomic, etc.), reducing genome stability also contributes to gene-level instability (Stevens *et al.,* 2011b). While this certainly has a micro-cellular evolutionary role in altering given systems, genome-level change plays a larger macro-cellular evolutionary role by creating new systems, and this system replacement achieved by genome-level change is essential for cancer evolution. Thus, genome-level information when profiling tumour subtypes and/or predicting drug response holds the highest clinical value.

Interestingly, many normal, healthy tissues display genome alterations; examples include liver polyploidisation, skeletal muscle, thyroid gland, blastocyst mosaicism, blood, urothelium and Purkinje neurons, as well as detected stochastic karyotypic changes as the result of environmental and physiological challenges (Biesterfeld *et al*., 1994; Celton-Morizur and Desdouets, 2010; Davoli and de Lange, 2011; Fragouli and Wells, 2011). Recently, based on these phenomena, it was suggested that CIN-mediated genome heterogeneity likely plays an important role for cellular adaptation (Horne *et al*., 2014). As a trade-off, cancer can be considered a price to pay for adaptation, as elevated

instability can also lead to various diseases (Heng *et al*., 2015). Thus, it is important to investigate the overall benefits/harms of CIN, as well as whether certain types or degrees of CIN (e.g. numerical CIN and complex structures) are more effective at breaking down system constraint en route to a cancer phenotype (i.e. the perfect storm) than other aberrations, which remain under system constraint and contribute to healthy, complex cellular and organ function.

One example of this is through studying a balanced (rather than highly aggressive) approach of cancer therapeutics. As cell death induction could inadvertently lead to CIN (Stevens *et al.*, 2011a; Liu *et al.*, 2014), it is crucial that efforts be made to prioritise cancer genome system constraint over the current clinical goal of maximising tumour cell death (i.e. through maximum tolerated doses). While initially effective (i.e. high cell death count), high-dose therapeutics induce genome chaos (Liu *et al*., 2014), resulting in increased genomic heterogeneity. This increase in evolutionary potential is coupled with the induced formation of aggressive outlier subpopulations (i.e. through genome reorganisation), which ultimately drive rapid tumour cell growth (Horne *et al*., 2015a; Horne *et al*., submitted). This process serves as a general mechanism of cancer drug resistance and presents a trade-off of the current maximum tolerated dose strategy, where early success (i.e. high initial cell killing) comes with long-term detriment (i.e. rapid relapse and drug resistance). The strategy of reducing CIN-induced genome heterogeneity fits well with some alternative therapies such as adaptive and metronomic therapies, which have shown promise in early trials and call for lower dosages than standard care (Kerbel and Kamen, 2004; Gatenby *et al*., 2009).

The CIN-mediated dynamic nature of the cancer genome also presents a problem for specific target-based therapies, which have been popular following cancer genome sequencing efforts (Horne *et al*., 2013). As there is no fixed cancer genome, driver genes can vary between cancer cells and switch roles at any given time. This poses a major challenge to the success of targeting therapies: as long as CIN is involved, administration of any agent will result in off-target effects despite initial high levels of cell death, while paradoxically initiating another round of CIN.

Finally, monitoring CIN within an evolutionary context can provide valuable information for both cancer research and treatment. We have demonstrated the utility of the NCCA/CCA index in understanding the evolutionary potential of the disease at a particular time. Measuring CIN in such a manner can help identify the evolutionary phase of the disease, and this information is useful in determining treatment administration and patient prognosis. For example, we could reduce tumour cell growth by applying constraint through careful dosage administration in order to maintain the less dynamic phase of cancer evolution. In other words, the key to more successful cancer treatment (i.e. improved patient quality of life and longer life span) is to slow down the micro-evolutionary phase without triggering genome chaos. Some important questions include should patients with high or low degrees of CIN undergo identical, aggressive treatment regimens? How can we use CIN in classifying patients into subgroups? And lastly, should we continue efforts to identify genes that can be linked to elevated CIN knowing that there are so many non-genetic factors that are also involved?

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ABSTRACT

EFFECTIVE DRUG TREATMENT INDUCES DRUG RESISTANCE THROUGH RAPID GENOME ALTERATION-MEDIATED CANCER EVOLUTION

by

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August 2016

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The central paradox associated with current cancer therapeutic strategies is initially effective treatment, which eliminates a high tumor cell count, consistently results in successful drug resistance. Mathematical and evolutionary modeling have previously suggested that therapeutic intervention could provide selective pressure for the expansion of resistant variants. Drug-related stress has been associated with genome chaos, a common phenomenon in cancer characterized as rapid, stochastic genomic fragmentation and reorganization. Since cancer represents an evolutionary process, analysis within the context of genome-mediated cancer evolution can shed light on this key problem of therapeutics. We propose that genomic change is a general response to therapeutics. Drug-induced karyotypic alteration has been linked with transcriptomic elevation, implying that drug-induced genomic change would paradoxically provide an advantage for cancer cells through an increase of genome heterogeneity or evolutionary potential for selection. In vivo and in vitro

models were tested using different therapeutic approaches, and surviving cells displayed altered karyotypes for each case. To determine whether drug-induced genome change could provide a long-term advantage to cancer cell survival, a karyotypically stable colon cancer cell line was treated with chemotherapy, and growth patterns were followed in a series of in vitro single-cell and population-based experiments. Outlier treated cells displayed faster growth rates than untreated cells, and population-based data support that these outliers may drive cancer progression post-therapy. This macro-evolutionary based, general mechanism of cancer drug resistance challenges the current therapeutic aim of maximizing cancer cell death and has great implications in the development and administration of future therapeutic strategies.

AUTOBIOGRAPHICAL STATEMENT

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EDUCATION

Ph.D. Molecular Biology and Genetics, Wayne State University, August 2016
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SELECTED PUBLICATIONS

Horne, SD et al. (2016). Effective drug treatment induces drug resistance through rapid genome alteration-mediated cancer evolution. Submitted.
Horne, SD et al. (2015). Chromosomal instability (CIN) in cancer. *eLS*, 1-9.
Horne, SD et al. (2015). Cancer genome evolution. *Transl Cancer Res*, 4.3, 303-313.
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AWARDS & HONORS

♦ First Place Poster Presentation, 1st Annual Graduate and Post-Doctoral Research Symposium, Wayne State University, 2016
♦ Thomas C. Rumble University Graduate Fellowship, 2015-2016, 2013-2014
♦ Wayne State University Summer Dissertation Fellowship, 2015, declined
♦ Broadening Experiences in Scientific Training Fellowship, 2015, declined
♦ Wayne State University External Fellowship Incentive Award, 2012
♦ Wayne State University Interdisciplinary Biomedical Sciences Fellowship, 2010-2012
♦ Wayne State University Dean's Enhancement Award for Undergraduate Academic Achievement, 2010

SELECTED CONFERENCE PRESENTATIONS

♦ Drug treatment fuels genome-mediated cancer evolution, 16th Annual Midwest DNA Repair Symposium, 2014.
♦ Genome chaos – an effective mechanism for cancer evolution, 4th Annual Graduate Midwest Research Symposium, 2013.
♦ Applying spectral karyotyping to monitor ART-associated genome-level alterations, 3rd Annual Michigan Alliance for Reproductive Technologies and Science Conference, 2012.