An Evolutionary and Ecological Investigation of Cancer

BY

MARK CASSIDY CRIDLIN LLOYD

B.S., Dickinson College 2000

M.S.M., University of South Florida 2008

THESIS

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Defense Committee:

Joel S. Brown, Ph.D.	Chair and Advisor
Roberta J. Mason-Gamer, Ph.D.	Biological Sciences
Emily S. Minor, Ph.D.	Biological Sciences
Robert A. Gatenby, M.D.	H. Lee Moffitt Cancer Center and Research Institute
Marilyn M. Bui, M.D. Ph.D.	H. Lee Moffitt Cancer Center and Research Institute

I dedicate this work to the world's cancer patients and those doctors and scientists who have dedicated their mindshare toward understanding and unravelling this disease.

It is important for us to remember all of those who we have loved who have passed and all of those who are currently struggling with this disease. These individuals have fueled my motivation to study cancer and novel avenues to understand this malady. I have found it to be critical to spend time with patients, to learn about them as people, their experiences and their struggles with the disease. I do not think it is sufficient to design and perform experiments in the laboratory. I believe it is necessary to walk the halls of the clinics. To eat lunch surrounded by patients and their families. To spend reflective time in the hospital's waiting rooms. These simple activities have powered my drive to study this disease- and it is for these patients that I dedicate this work.

ACKNOWLEDGEMENTS

This work was accomplished as a team effort. First and foremost, I wish to acknowledge my wife, my best friend and my everything, Jennifer Schauble Lloyd. Jen made this work possible. When I enrolled in this program three and a half years ago I had already undertaken many other life roles as well. I am a husband, a father, a full-time cancer researcher and the founder of an emerging cancer diagnostics company. One does not perform so many tasks on their own. Jen gave me the freedom, the time, the support and the encouragement to follow my dreams, to aspire beyond conventional standards and to grow as a scientist and as a person. For that- I can never fully repay her. It is with my utmost gratitude that I thank Jen for making this learning possible.

I would also like to thank my daughter Maya. Maya was three years old when I enrolled in this program. We lived in Tampa and I studied in Chicago. The travel schedule was intensive and being away from my family was a great sacrifice that was shared among all of us. I want to thank Maya for sharing me and her time with me with this program.

I thank my parents, Bob and Debbie Lloyd, who raised me to be ambitious. They taught me to see opportunity and to follow my dreams. They have always been supportive of me, even when my decisions were poor. They taught me that I could take risks if I was willing to be responsible for my actions. This gave me, over the years, the confidence that I could achieve my goals, even if they were outside usual norms.

I thank the H. Lee Moffitt Cancer Center and Research Institute, its doctors, researchers, staff and administration. It is with great admiration that I recognize Moffitt for its vision to allow me to exceed the normal limits of work, study and personal commitments. Without their support I would not have been able

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LIST OF ABBREVIATIONS

+: positive

-: negative

AGE: advanced glycation end-products

ANOVA: analysis of variance

CAIX: carbonic anhydrase 9

CAXII: carbonic anhydrase 12

CC3: cleaved caspase 3

CCD: charge-coupled device

CD34: CD34 molecule of cell surface gylcoproteins

CIITA: class II transactivator

Cy5: cyanine dye 5

DCIS: ductal carcinoma in situ

ER: estrogen receptor

ER-: estrogen receptor negative

ER+: estrogen receptor positive

ESS: evolutionarily stable state/ strategy

FFPE: formalin fixed, paraffin embedded

GLO1: glyoxalase-1

GLUT1: glucose transporter 1

H-Score: histological score

Her2: human epidermal growth factor receptor 2

HIF1α: hypoxia inducible factor 1 alpha

HLA: human leukocyte antigen

HLA-DR: human leukocyte antigen- D related

HMGB-1: high-mobility group box 1

H&E: hematoxylin and eosin

IFN-γ: interferon-gamma

IL: interleukins

K: maximal carrying capacity

KI67: protein also known as MKI67

LDL: low density lipoproteins

LiDAR: light detection and ranging

MAKP: mitogen activated protein kinase

MGO: methylglyoxal

m= migration rate

MGO: methylglyoxal

mm: millimeter

MMB: Marilyn M. Bui (committee member and pathologist)

MMP: matrix metalloproteinases

NA: numerical aperture

NO: nitric oxide

PBS: phosphate buffered saline

PE: phycoerythrin

r: maximal growth rate

RADAR: radio detection and ranging

RAGE: receptor for advanced glycation end-products

RGB: red, green, blue

RPMI: Roswell Park Memorial Institute cell growth medium

RSV2: melanoma cell line with an empty expression vector

SLC2A1: solute carrier family 2 facilitated glucose transporter member 1

TNF: tumor necrosis factors

VEGF: endothelial growth factors

WSI: whole slide images

x= magnification times

μm: micron

SUMMARY

How does natural selection optimize cancer cell population dynamics? To answer this question I devised six unique approaches described in the chapters of this dissertation. The first question to be addressed was whether or not subpopulations of morphologically distinct cells could be identified spatially? Morphologically distinct cancer cells were identified in spatially predictable formations which further raised the question- does this represent distinct evolutionary strategies to optimize fitness in a given environment? Here I investigated the possibility that intratumoral evolutionary dynamics based on environmental selection forces could, in fact, regularly achieve a local fitness maximum.

Next, I aimed to understand if pathologic feature analysis integrated with spatially explicit ecological methods could elucidate regional variations in environmental selection forces and phenotypic adaptations? I studied how quantitative, spatially-explicit methods developed in landscape ecology could be leveraged to define the underlying heterogeneous biological processes in tumors within individual patients.

Furthermore, it was important to learn if there are clinically impactful cellular traits selected for in local microenvironments? Here I focused on the hypothesis that estrogen receptor (ER) expression is a successful adaptive strategy only if estrogen is present in the microenvironment. Since the dominant source of estrogen is blood flow, I hypothesized that, in general, intratumoral regions with higher blood flow would contain larger numbers of ER+ cells when compared to areas of low blood flow and in turn necrosis. I concluded that ER expression can be understood as a Darwinian process and linked to variations in estrogen delivery by temporal and spatial heterogeneity in blood flow. This correlation suggested strategies to promote intratumoral blood flow or a cyclic introduction of estrogen in the treatment schedule could be explored as a counter-intuitive approach to increase the efficacy of anti-estrogen drugs.

Then, I asked what mechanisms of the ecological competition exist between cancer and normal cells? I studied melanoma cells which express high levels of HLA (human leukocyte antigen) class II. This cell

surface, antigen-presenting protein is an anomalous phenotype among solid tumors, yet there has never been a satisfying explanation for how this HLA class II positive phenotype is related to tumor development. I co-cultured melanoma and CD4-positive, labeled, Jurkat-C T-cells. The melanoma cells were transformed with an expression vector for class II transactivator (CIITA), the obligate HLA class II gene transactivator. I then assayed for the transfer of label to the melanoma cells. The result included CIITA expression facilitated engulfment of the T-cell material but not material from B-cells. This suggests a possible mechanism for HLA class II positive melanoma cells in blunting an anti-tumor competitive response and suggests a possible target for melanoma therapy.

Next, I asked how tumor evolution and the integrity of the tissue environment can impact cancer cell invasion and disease progression. In this study I created a model to map out multiple metabolite wastes known to be an unintended consequence of tumor growth. Specifically, after careful study, I targeted methylglyoxal's (MGO) role in cancer development to investigate tumors' production and use of these toxic metabolites and the impact of these metabolites may have on tumor evolution, the integrity of the tissue environment, and, ultimately, the patient.

Finally, with a more holistic view of the patient, I began to look beyond the primary tumor toward selection forces and adaptations which may provide new insights into cancer cell metastasis. I asked the question, can the principles and concepts from invasion ecology meaningfully contribute to a novel framework for understanding the metastatic cancer process and introduce new ideas for clinical treatment? Within the biotic mix of any given neoplasm are clusters of heterogeneous tumor and normal cells that compete for abiotic resources such as space and nutrients. Given the high competition and limited resources, it is reasonable to expect tumor cells to use a strategy of dispersal and colonization of secondary sites. I described concepts of invasion ecology, frame metastasis within existing theories of ecological invasion and proposed several different strategies which heterogenic tumor cells may employ to successfully populate distant body sites. I concluded that metastases is an asymmetric nesting process and at least

five major types of tumor cell strategies are involved in the metastatic cascade which I termed “ecological engineer”, “ecological pioneer”, “penguin”, “treasure-seeker” and “canoeist”.

In summary, the six scientific chapters of this dissertation were crafted to address my fundamental interests of the evolutionary ecology of cancer. I define cancer as a speciation event where a metazoan spins off a single cell protist. Furthermore, I generally define cancer as a disease of evolution from natural selection. Most frequently I have chosen a technical approach which leverages imaging and image analysis to investigate adaptations to the fit of form and function. I believe these ideas and approaches are unique, challenge the current paradigm of thinking in oncology, and will facilitate a rich research program focused on my key question: **‘how does natural selection optimize cancer population dynamics?’**

I. INTRODUCTION:

Overview

I am an evolutionary cancer ecologist. I ask the question- How does natural selection optimize cancer population dynamics? I believe that nature is our best teacher. My research investigates individual cancer cells and cancer cell populations using submicron imaging to study evolutionary and ecological dynamics of cancer. More specifically, I leverage my prior interests and expertise to interrogate spatial distributions of cell populations using tools of digital whole slide imaging of histological samples in and around malignancies.

One, if not the most commonly used, definition of cancer is 'a disease of the genes' [Vogelstein 2004]. I define cancer as a speciation event where a metazoan spins off a single cell protist. I believe cancer begins as a series of somatic mutations with limited Darwinian evolution at its initiation but then, at the point of cancer establishment within a patient, rapidly becomes a disease of evolution from natural selection [Melo 2007].

I propose to investigate how Darwinian dynamics, as defined as the simultaneous evolution of populations and strategies on a continuous adaptive landscape, applies to cancer [Vincent 2005]. I am interested in investigating how and why intratumoral spatial heterogeneity of cancer cell populations, although sometimes initiated by random mutations, must be governed by identifiable Darwinian dynamics [Santos 2006, Gillies 2012]. **My central hypothesis is that the physical morphology of cancer cells, which can be observed and quantified, provides deep insight into adaptations governed by variations in selections forces within local microenvironments.** Furthermore, I submit that an evaluation of the evolution of cancer's intratumoral heterogeneity is not chaotic or unpredictable, but can be understood by identifying regional variations in selection forces and adaptive strategies [Lloyd 2014].

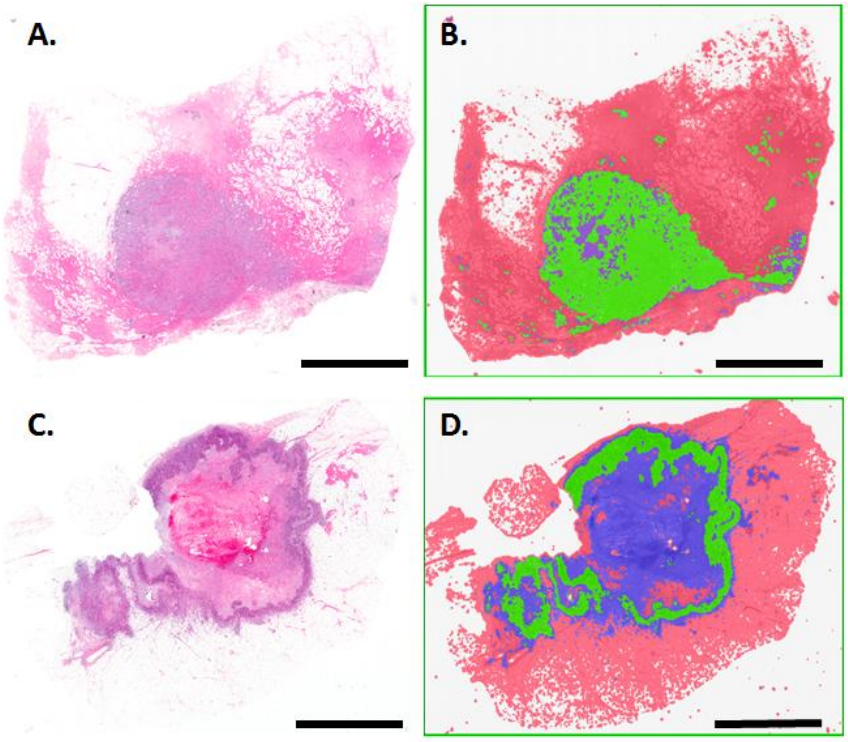

Figure 1.1 demonstrates morphological changes in variable spatial environments. Regional morphological quantification of the standard of care diagnostic slides stained with hematoxylin and eosin (H&E). Regions of viable tumor were selected via histological pattern recognition software (Genie; Aperio) in green. Normal tissues are classified in red and necrotic tissues are classified as blue. In the top panel A) and B) and ER+ sample is presented in comparison to C) and D) in which an ER- sample is shown. Scale bar = 5mm

I propose to use this general research thrust to begin to address the following key questions which were formed into the dissertation chapters which follow:

1. Can subpopulations of morphologically distinct cells be identified spatially? Does this represent distinct evolutionary strategies adapted to optimize fitness in a given environment?
2. Can pathologic feature analysis integrated with spatially explicit ecological methods elucidate regional variations in environmental selection forces and phenotypic adaptations?
3. Are clinically impactful cellular traits selected for in local microenvironments?
4. What mechanisms of ecological competition exist between cancer and normal cells? Can they be exploited for novel therapy design?
5. How does tumor evolution and the integrity of the tissue environment impact cancer cell production and use of toxic metabolites? This chapter extends our investigation of cell metabolism to consequences for the tumor and the body.
6. Can the principles and concepts from invasion ecology meaningfully contribute to a novel framework for understanding the metastatic cancer process and introduce new ideas for clinical treatment?

In my last 12 years as a staff scientist at the H. Lee Moffitt Cancer Center and Research Institute I have had a great deal of exposure to the concept of personalized medicine. When I was first introduced to personalized medicine, which is defined as using patient-specific metrics to provide an optimal cancer therapy customized for each individual patient [Hamburg 2010], I was both interested and then quickly disappointed. Treating the right patient at the right time with the right therapy was exciting. However, the

approach being used at Moffitt then, and most major cancer centers worldwide today, is focused almost solely on genomic approaches [Ginsburg 2001]. This approach struck me as grossly incomplete. This is because it does not take into account the morphology of cancer- and in turn, form, fit and function [Dietel 2006]. The goal of my doctoral dissertation is to provide the field with an alternative view of cancer as a species struggling to survive. Finally, I aim to explore how this research direction can mature to exploit predictable evolutionary processes to treat cancer in a significantly more efficient way [Greaves 2012].

To state that I view cancer as a species, or small number of species, I must also define speciation. I began by posing the following question- why isn't there a single definition of species? Is it because different definitions make sense in different situations (i.e. biological species concept in biologically classifying sexually reproducing organisms or phylogenies in understanding a species' genealogic trajectory)? We use species as ways for humans to organize organisms into groups that 'make sense'. In the context of all organisms, and cancer cells are no different- the 'Strategic Speciation Concept' is, in my view, the most accurate and inclusive way to define species [Vincent and Brown 2005]. This concept focuses on distinct strategies towards fitness optimization. Cancer initiation rises from mutations. But cancer evolution is a result of strategy refinement toward fitness maxima. So while 'taxonomists further group species into genera, families, orders, and kingdoms, while ecologists group species into higher structures such as communities and ecosystems' [Mallet 2001] - what do oncologists do? Cancer biologists and physicians group cancer into progressive stages. But I view this as grossly overly simplistic.

Progression of disease does not capture the true trajectory of a cancer's morphological, behavioral or even genetic changes [Mcneal 1986; Bombonati 2011]. The descriptions of cancer today- well, moderately and poorly differentiated- does not adequately address natural selection or fitness [Merlo 2006]. The strategic speciation concept in cancer is most similar to the morphological species concept, in that the way cancer looks defines it (albeit still not into speciation events) [Vincent and Brown 2005]. This is the main approach I have taken in measuring the morphological similarities and differences in cancer cell morphology. The strategic speciation concept also touches on the phylogenetic view of speciation in that the genealogic

trajectory of the cancer as it progresses from in situ to invasive disease is addressed. Yet neither of these species concepts are complete in describing intratumoral cancer speciation events as I see them. Furthermore, the ecological species concept of occupation of ecological niches provided by van Valen [van Valen 1976] has a direct role in understanding the adaptive landscape in which the strategic species concept resides. Finally, the competitive species concept [Rosensweig 1978] which includes a species' competitors within the adaptive landscape supports the view that natural selection can drive speciation, yet is not in my view, as complete as the broader concept of strategic species concept.

Thus, I define species as populations of individuals with a distinct strategy in an adaptive landscape. Natural selection is the key to the evolution of each strategy towards an optimum (or toward extinction). This can be viewed as survival of the fittest. And in oncology, my definition of evolving 'optimal cancers'.

My World View

The work herein is colored by my own personal world view. I take this opportunity to describe my individual perspective on this subject and its study. I grew up in a rural area on a horse farm in Virginia. I had the opportunity to spend my childhood days waist deep in the Shenandoah River on the North side of our property or climbing in the foothills of the Blue Ridge mountains to the East. Nature was my playground and I made the most of it. I achieved a healthy respect for the natural world and a wonder at its many secrets. As I grew, my appreciation of my natural surroundings matured.

I also had a number of important travel opportunities in my early and young adult life which began to mold my world view. My family and I traveled frequently when I was very young- and often far off the beaten path. We ventured into the heart of the Yucatan peninsula in Mexico and deep in the jungles of Columbia and Venezuela. I climbed in the Andes, traversed the Panama Canal and ascended glaciers on the west coast of Canada. In my young adulthood I drove across the country with my wife to-be, backpacked Europe, traveled across West Africa by tro-tro (shared van) and hitchhiking, hiked volcanos in Guatemala and El

Salvador, attended a wedding in China, honeymooned in the South Pacific, adventured in Jamaica and traversed over 1000 miles across the Atlantic Gulfstream by sailboat. As an adult of almost 40 years, I now travel to Europe for business and pleasure. In 2014 I opened a research and development center for my company in Bangalore, India. I consider myself a traveler. I mention my travel as background to what I believe has molded a global view of my surroundings. I recognize the depth of the many environments around the world and the social and cultural differences which have grown up in each place I visit. These are important observations which I bring to my investigations as a scientist.

I have not always been the most well-read, the student to score the highest marks or a good "test taker". I have, however, always thrived in academia. I flourish, in part, due to my intuition and ability to see opportunity. I believe in academia one can be first or one can be better- to succeed. I have always had the vision to see opportunity and the courage to be the first to attempt something. But I have also always had the best of mentors. As a child, my parents, my horse racing trainers (riding race horses was an exciting and rare opportunity I embraced throughout much of my young adulthood) and in high school, my teachers, were some of the most generous and exceptional mentors a young person could dream of. Currently, my advisor, Joel and cancer research mentors, Bon and Marilyn have embraced that role. They have helped me mature into the scientist I am becoming.

I believe my view of the world as a dazzling ball of energy with a near endless assortment of different surroundings and adaptions to those environments is a macrocosm of my study of cancer. I view a patient as the world for cancer. A self-enclosed system of a plethora of different natural environments- each with its own opportunities and challenges. I view cancer cells as species of inhabitants within that bounded organism. I view therapy as a perturbation of that system intended to irradiate the cancer from the system, but with many unintended consequences, including the potential for changing strategies of the surviving cancer cells themselves as they adapt to changes in their environment. I believe these parallels between the natural world and cancer biology may allow investigators to tap the knowledge and resources of evolutionary ecology to enrich their own study of cancer. This view stems from my early formed relationship

with nature, my exposure to the world and my concern that cancer biology today is too focused on mechanism, and may not be seeing the forest for the trees. Herein this dissertation I provide an alternative direction to study cancer- from the world view of an evolutionary cancer ecologist.

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II. DARWINIAN DYNAMICS OF INTRATUMORAL HETEROGENEITY: RANDOM MUTATIONS OF VARIABLE SELECTION FORCES

Abstract

Spatial heterogeneity in the molecular properties of cancer cells is generally attributed to branching clonal evolution driven by random mutations that accumulate throughout the tumor's history. This view rests on an implicit assumption that cancer cells can always acquire mutations that increase proliferation and, therefore, can never evolve to a fitness maximum. Here we investigate the possibility that intratumoral evolutionary dynamics could, in fact, regularly achieve a local fitness maximum. Modeling these dynamics using evolutionary game theory, we demonstrate that tumor populations in a stable environment will homogeneously converge to the fittest phenotype so that cellular spatial heterogeneity results from regional variations in environmental conditions driven, for example, by alterations in blood flow. Model simulations specifically predict a common spatial pattern in which cancer cells at the tumor-host interface exhibit invasion-promoting, rapidly proliferating phenotypic properties while cells in the tumor core maximize their population density by promoting tissue infrastructure such as angiogenesis. We tested model predictions through detailed quantitative image analysis of phenotypic spatial distribution in histological sections of 10 patients with invasive breast cancers. CAIX (F-Ratio=255.77, P<0.001), GLUT1 (F-Ratio=22.08, P<0.001) and KI67 (F-Ratio=73.58, P<0.001) were significantly upregulated in the tumor edge consistent with an acid-producing invasive, proliferative phenotype. Cells in the tumor core were 20% denser than the edge (F-Ratio=15.39, P<0.001) and exhibited upregulation of CAXII (F-Ratio=329.27, P<0.001), HIF1α (F-Ratio=13.40, P=0.001) and Cleaved Caspase 3 (F-Ratio=45.23, P<0.001) consistent with a more static and less proliferative phenotype. Similarly, vascularity (F-Ratio=22.48, P<0.001) was consistently lower in the tumor center compared to the tumor edges. Immune (lymphocytic) response to tumor antigens was greater in the tumor edge but this was not statistically significant (F-Ratio=0.057, n.s.). We conclude that at least some intratumoral heterogeneity in the molecular properties of cancer cells is governed by predictable regional variations in environmental selection forces.

Introduction

While patient-specific, precision therapy remains an important goal in oncology, treatment strategies based on static and non-spatial data can be limited as somatic evolution continuously alters the tumor environments and cell populations over space and time [Longo 2012]. For example, recent studies demonstrate significant intratumoral spatial heterogeneity in the molecular properties of cancer cells in several tumor types [Gerlinger 2010; Sottoriva 2013; Yachida 2010]. These regional variations are widely recognized as evidence of intratumoral evolution but the proposed dynamics typically focus on random acquisition of mutations that confer a fitness advantage resulting in a "selective sweep" [Greaves 2012; Merlo 2006] by the new population. An important clinical implication of this conventional model is that intratumoral molecular heterogeneity, because it is dependent on stochastic accumulation of mutations, must be fundamentally unpredictable.

An implicit assumption of conventional models of intratumoral evolution is that cancer cells do not achieve a fitness maximum. That is, cancer cells can indefinitely experience new mutations that increase their fitness allowing a new population to emerge even within a static environment. In contrast, we note that very different dynamics will results if tumor cells, like most species in nature, rapidly evolve to local fitness maximum [Gatenby 2011]. Critically, once cancer cell reaches a fitness maximum, no heritable change can further increase its fitness. In fact, under such conditions, the population will tend converge to a single dominant phenotype resulting in decreasing heterogeneity. Thus, in this alternative model of cancer evolution, spatial variation of phenotypes must be the result of local variations in environmental factors that may result from, for example, spatial and temporal heterogeneity in blood flow, which is almost invariably observed in cancer imaging. Importantly, in contrast to the conventional model, this model requires molecular characteristics of cancer cells to be non-random. Rather, they are governed by regional variations in environmental selection pressures and, therefore, predictable with sufficient understanding of the local Darwinian dynamics [Alfarouk 2013].

Here we frame this hypothesis using mathematical models from evolutionary game theory [Michelson 1987; Aktipis 2013]. The quantitative methods extend prior work that applied classic evolutionary trade-offs between fecundity and survivorship. That is, we propose cancer cells, like all evolving organisms, can invest resources to maximize fecundity or survivorship but not both [Gatenby 2014]. This Darwinian trade-off manifests in cancer cells as two tumor cell types roughly correspond to what is known as r and K selection [Gatenby 2003] where "r" refers to a species with maximal growth rate (capacity to grow at low population densities) as opposed to "K" referring to a species that maximizes its carrying capacity (capacity to maintain growth at high population densities).

The model can develop complex spatial population dynamics depending on spatial and temporal variations in blood flow and other components of the host response. However, we find that the models consistently predict one property of the tumor ecology – that the cancer cells at the tumor-host interface will demonstrate phenotypic properties that are consistent between tumors but very different from the properties of cells deeper within the same tumor. These predictions are quite similar to those observed in the leading edge of invasive species in nature. For example, cane toads and sparrows from the invasive fronts in Australia and Kenya, respectively, exhibit phenotypic properties that optimize their ability to invade adjacent unoccupied territories not found in individuals from already colonized regions.

We then tested the model through detailed, quantitative analysis of spatial distribution of phenotypic properties in histological sections taken from 10 patients with clinically invasive breast cancers. We compared edge and center regions of each tumor for habitat variables such as vascularity and lymphocyte infiltration. We then compared the density of the tumor cells at the edge and center locations and the molecular properties of tumor cells inhabiting these regions.

Methods

All clinical components of the study were completed with the approval of the University of South Florida Institutional Review Board. Participant's written consent was not obtained because all personal health information was deidentified and analyzed anonymously. The Moffitt Scientific Review Committee and University of South Florida IRB committee both approved this protocol (MCC 16511).

Mathematical Model

We investigate a mathematical model from evolutionary game theory of habitat heterogeneity (Brown and Pavlovic 1992) in which we envision two habitats: the core of the tumor versus the tumor edge. We assume that cancer cells can evolve while normal cells do not. However, normal mesenchymal cells retain phenotypic plasticity and may be influenced and/or co-opted by the tumor cells to generate a tissue infrastructure that favors cancer growth. Within a habitat we assume that the cancer cells compete for limiting resources but do not interact directly with cells in the other habitat. Indirectly, however, their habitats do interact via migration where a fraction of the population from each habitat actively moves into the adjacent habitat or find themselves in that habitat as the edge of the tumor either recedes, expands or shifts location.

We imagine an evolutionary strategy that represents a tradeoff between capacities to produce Carbonic Anhydrase (CA)XII versus CAIX. CAIX and XII are extracellular enzymes that catalyze the reversible hydration of CO_2 to bicarbonate and a proton:

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$$

CAIX is a transmembrane glycoprotein whose catalytic domain faces the extracellular milieu [Gatenby 2003]. CAXII has a similar overall secondary structure and orientation to CAIX, although missing the PG-like domain. CAIX sets the extracellular pH at 6.8 while CAXII sets the extracellular pH at 7.4. We use this difference in extracellular pH set point as markers for phenotypic strategy. It is also reported that CAIX is a poor prognostic indicator and CAXII is a positive prognostic indicator in breast cancer [Gillies 2008].

The buffering and habitat modulating properties of CAXII promotes or are associated with higher carrying capacity (K) and lower maximum proliferation rates (r) – such a species emphasizing CAXII is "K-selected". The acid tolerating properties of CAIX promote or are associated with resistance to the immune system, degradation of normal cells, and higher proliferation rates – such a species is "r-selected". We scale the heritable strategy, u, to range from $u = 0$ (maximum carrying capacity K and minimum growth rate r) to $u = 1$ (minimum K and maximal r).

Via competition within habitats and migration between habitats, the tumor cells engage in an evolutionary game in which an individual's fitness, $G(u, \boldsymbol{u}, \boldsymbol{x_A}, \boldsymbol{x_B})$, depends upon its strategy, u, the strategies of the other tumor cells, $\boldsymbol{u}$, and population sizes of tumor cells in the interior (A) and edge (B) of the tumor, $\boldsymbol{x_A}$ and $\boldsymbol{x_B}$, respectively. The fitness generating function is given by

$$G(u, \boldsymbol{u}, \boldsymbol{x_A}, \boldsymbol{x_B}) = pF_A + (1-p)F_B.$$

The fitness of a focal individual in a habitat $\mathrm{i} = \mathrm{A}\ \mathrm{or}\ \mathrm{B}$ of species j is a function of its strategy u and the current density of individuals within that habitat $\mathrm{x_i}$ given by

$$\mathrm{F_i(u, u, x_i)} = \mathrm{r_i(u)}\left(\frac{\mathrm{K_i(u)} - \sum_{\mathrm{j}} \mathrm{x_{ij}}}{\mathrm{K_i(u)}}\right) - \mathrm{d_i},$$

where $\mathrm{d_i}$ is an extrinsic mortality term not built into the logistic growth due to ecological properties of the habitat. The individuals' strategy u within a habitat affects both the logistic growth rate and carrying capacity given by

$$\mathrm{r_i(u)} = \mathrm{r_{i0}} \exp\left(-\frac{(\mathrm{u}-1)^2}{\sigma_{\mathrm{r}}^2}\right) \quad \text{and} \quad \mathrm{K_i(u)} = \mathrm{k_{i0}} \exp\left(-\frac{\mathrm{u}^2}{\sigma_{\mathrm{K}}^2}\right),$$

where $\mathrm{r_{i0}}$ is the maximum growth rate of each habitat $\mathrm{i} = \mathrm{A}\ \mathrm{or}\ \mathrm{B}$ and $\mathrm{k_{i0}}$ the maximum carrying capacity of each habitat $\mathrm{i} = \mathrm{A}\ \mathrm{or}\ \mathrm{B}$. σ_{K}^2 and σ_{r}^2 are constants characterizing the Gaussian penalty due to strategy to $\mathrm{r_i(u)}$ and $\mathrm{K_i(u)}$.

A strategy u will converge on a distribution of individuals among habitats such that the strategy has the same per capita growth rate in each habitat. Equilibrating $\frac{\partial x_{ij}}{\partial t}$ and substituting $q = x_A / x_B$ results in

$$q = \frac{(F_A - F_B + m_B - m_A) + \sqrt{(F_B - F_B + m_A - m_B)^2 + 4 m_A m_B}}{2 m_A}.$$

Therefore the frequency with which a strategy u will eventually experience habitat A for any fixed biotic environment is given by

$$p(u, u, x_A, x_B) = \frac{q}{(q+1)}.$$

The evolutionary dynamics of the cancer cell strategies can be visualized on an adaptive landscape. This landscape plots G versus the strategy of the focal individual, u. The adaptive landscape is fixed for a given tumor population with its associated strategies and population sizes. But, as the populations' strategies evolve (evolutionary dynamics) given by

$$\frac{\partial u}{\partial t} = c * \frac{\partial G}{\partial u}$$

where c is a constant that scales the speed of evolutionary change and their associated population sizes change (ecological dynamics) given by

$$\frac{\partial x_{ij}}{\partial t} = x_{ij} * \left[F_i(u_j, u, x_i) - m_i x_{ij} + m_l x_{lj}\right], l \neq i.$$

where m_i is the per capita migration rate of individuals from habitat i to the alternate habitat l for $l \neq i$, the landscape also changes. Hence the landscape itself is dynamic in response to the Darwinian dynamics of strategies and population sizes (Vincent and Brown 2005). At any time and point along this landscape the population will evolve "uphill" until it reaches a convergent stable point – at this point, the slope of the landscape is zero ($\partial G / \partial v = 0$), and the population sizes equilibrate so that fitness is 0 ($G = 0$). This convergent stable point can either be at a maximum or minimum of the adaptive landscape (Apaloo et al.

2009). If at a maximum, then the cancer has evolved to its evolutionarily stable strategies (ESS) and such a state will be both ecologically and evolutionarily persistent. If at a minimum, then the cancer cell population is under strong disruptive selection and it should “speciate” into two distinct clades that diverge and evolve to occupy distinct niches seen as distinct peaks of the adaptive landscape (Cohen et al. 1999).

Further methods, details and theories of the game theory model and image analysis strategies may be found in Supplemental material.

Case selection

Following approval by the Institutional Review Board, ten patients with formalin fixed and paraffin embedded (FFPE) blocks of diagnosed invasive ductal breast carcinoma were retrospectively examined. Cases were selected by a practicing pathologist (MMB) to include five each of the three Nottingham score grades.

Histology

Sectioning: The Tissue Core at Moffitt located each FFPE block and 4µm serial slides from each patient were sectioned using standard histotechnique.

Immunohistochemical staining

Slides were stained using a Ventana Discovery XT automated system (Ventana Medical Systems, Tucson) as per manufacturer's protocol with proprietary reagents. Slides were deparaffinized on the automated system with EZ Prep solution (Ventana) and the following reagents and incubation times:

- CD34 (mouse monoclonal CMA334; Cell Marque, Rocklin, CA) for 16 minutes + the OmniMap anti-mouse secondary antibody for 12 minutes + Ventana ChromoMap for detection
- KI67 (rabbit primary antibody 790-4286; Ventana) for 16 minutes + anti-rabbit secondary for 16 minutes + OmniMap for Detection

- CAIX (rabbit primary antibody #ab15086; Abcam, Cambridge, MA) at 1:500 in Dako diluent incubated for 32 minutes + OmniMap anti-rabbit secondary for 20 minutes + ChromoMap.
- CAXII (rabbit primary antibody #HPA008773; Sigma, St. Louis, MO) at 1:75 in Dako diluent for 32 minutes + OmniMap anti-rabbit secondary for 20 minutes + ChromoMap.

Each set was counter stained with hematoxylin then dehydrated and coverslipped per standard histological protocol.

Imaging and Analysis

Image acquisition: Stained slides were digitally scanned using the Aperio (Vista, CA, USA) ScanScope XT high-throughput slide scanning instrument (200x/0.75NA objective with a rate of 2-3 minutes per slide via Basler tri-linear array).

Segmentation: Histology pattern recognition technology used included both Aperio's GENIE® software and Definiens (Munich, Germany) TissueStudio™ v3.0 to identify tumor regions of interest [26, 27]. Regions of the tumor edge were defined as areas within 1mm of the tumor-host interface and tumor center regions were defined as any area deeper than 1mm of the tumor-host interface. For each measurement a 500µmx500µm subregion was randomly selected using a custom Matlab (R2014b) script. Three subregions were used for each analysis of the center or edge regions for each patient sample. Furthermore, single cells were identified as tumor and mesenchymal regions, respectively, by identifying the nuclei and growing cell simulations 5µm. The classified nuclear and cytoplasmic subcellular compartments were evaluated independently for biomarkers which localize to a specific cellular region. Intensity thresholds from each biomarker were determined by the study pathologist (MMB) and retained consistently for each patient set.

Results

Cancer adaptive landscapes and Intra-tumoral evolution

While tumors likely possess a large number of ecological niches, our model simulations focused on just two: 1. The tumor-host interface in which tumor cells compete primarily interact with elements of normal tissue including the predatory effects of the immune response and normal tissue infrastructure such as intact blood vessels and 2. The interior in which tumor cells compete with each other and must actively promote formation of the mesenchymal infrastructure required to support the population.

If the population of tumor cells is well mixed between edge and interior of the tumor (high migration rate, m), then evolution promotes an ESS that is a single clone (Figure 2.1).

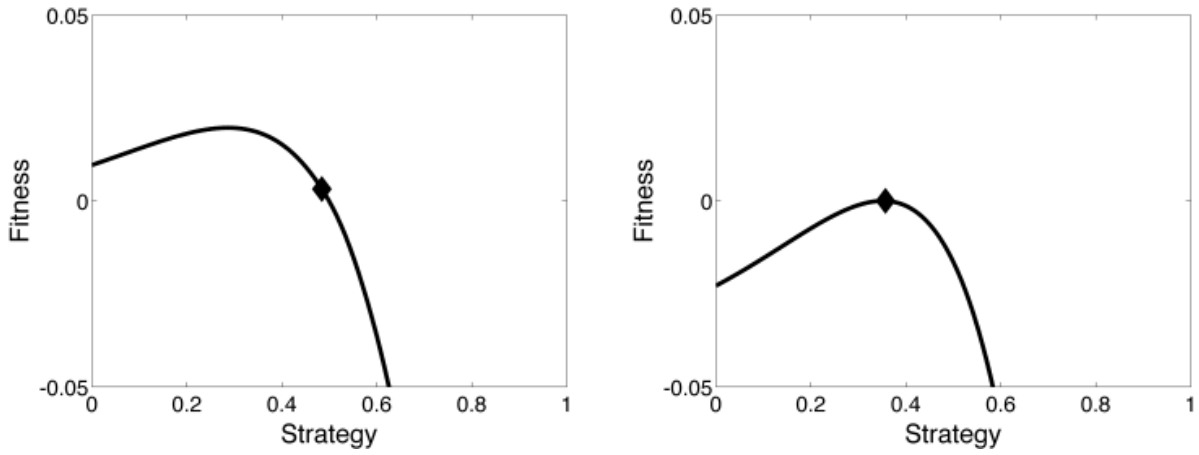

Figure 2.1 Evolution of a population of cells in an environment where the migration rate is high (m = 0.1). The initial population begins with a strategy u=0.5 (left panel). Evolutionary dynamics will cause this population's strategy to climb the adaptive landscape. Through both the ecological and evolutionary dynamics an ESS is achieved at a strategy of u=0.3564 (right panel).

This clone possesses a generalist strategy balancing the need for a higher r when facing the edge habitat and a higher K when facing the interior. No matter the starting strategy of the population, it will evolve towards the same peak of the adaptive landscape. We expect this outcome when either the spatial heterogeneity of habitat types is very fine-grained, or the cells are highly motile and frequently move from one habitat to the next (which itself could be a response to environmental selective pressures [Aktipis et

al., 2012]). For larger more advanced tumors, we would expect edge and interior habitats to be more coarse-grained, and the likelihood of a given tumor cell moving from one to the other to be relatively small on a per 8-24 hour basis (the likely unit of time in our model).

If the migration rate is small, an axis of heterogeneity describing the edge to interior of the tumor can result in the speciation of a single clonal cancer lineage into two distinct phenotypes specialized to exploit different regions of tumor heterogeneity (Figure 2.2).

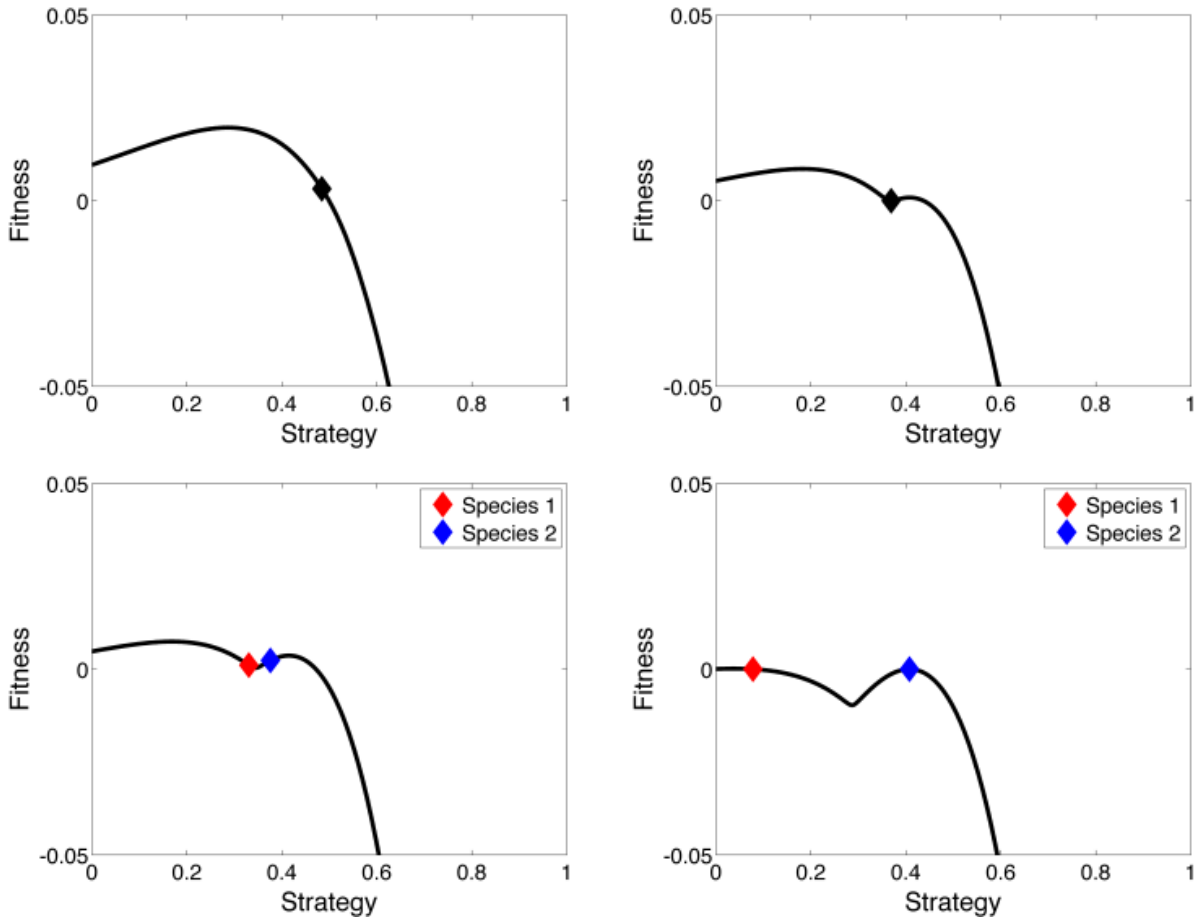

Figure 2.2 Evolution of a population of cells in an environment where the migration rate is low (m=0.001). Again, the initial population begins with a strategy of u=0.5 and begins to climb the adaptive landscape (upper left). Instead of achieving a peak, the population actually evolves to an evolutionarily stable minima of the landscape at u=0.3677 (upper right). Disruptive selection causes the single population to diverge into two separate species. The one being K-selected is shown as species 1 in red and evolves to an ESS of u=0.0774. The other being r-selected is shown as species 2 in blue and evolved to an ESS of u=0.4074 (bottom panels).

If the tumor starts with a single evolving population of cancer cells, then these cells evolve up the slope of the adaptive landscape. But instead of achieving a peak, they actually evolve to an evolutionarily stable minima of the landscape. At this point disruptive selection should promote speciation and the divergence of separate tumor cell types. The one being K-selected (CAXII) and the other r-selected (CAIX). While some spatial overlap will occur between the two types, the former will predominate in the interior of the tumor and the latter at the tumor's edge.

More generally the model shows how the grain-size of habitat heterogeneity and the motility of tumor cells will determine whether tumor heterogeneity promotes generalist versus more specialist tumor "species". Figure 2.3 shows how low migration rates promote speciation and divergent strategies among the tumor cells. As the migration rate increases, the values of the two strategies comprising the ESS begin to converge and do so at a critical threshold value of migration. At higher rates of migration above this threshold, the ESS is a single species with a generalist strategy (Figure 2.3).

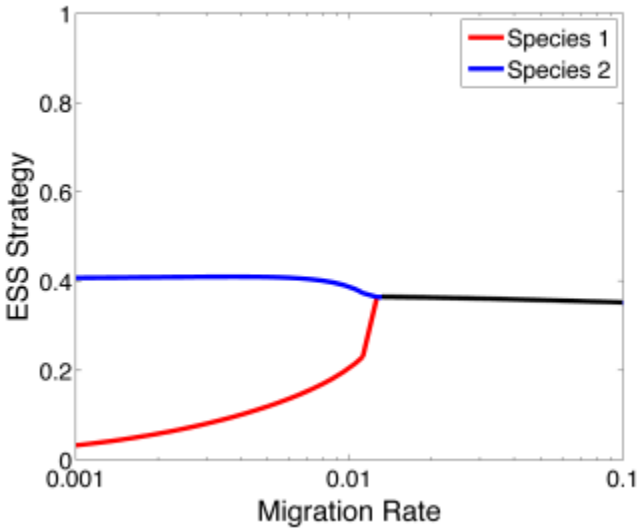

Figure 2.3 Evolutionary stable strategies vs. the migration rate m. Speciation into two distinct strategies occurs at low migration rates (m < 0.012). At high migration rates (m> 0.012) the values of the two strategies converge to a single species with a generalist strategy. The dynamics of m = 0.1 are shown in Figure 2.1 and the dynamics of m = 0.001 are shown in Figure 2.2.

In summary, the model simulations demonstrate that selection forces in the tumor core favor tumor cells that maximize carrying capacity by promoting angiogenesis and aggressively competing for limited resources. Conversely, the tumor cells at the leading edge (i.e. the tumor-host interface) maximize their local fitness by investing resources in invasive strategies that permit them to acquire resources through co-opting normal vessels and other host mesenchyma even at the expense of a potentially higher death rate due to host response. Thus, in general our models predict "engineering" phenotypes will dominate the tumor core while cells at the leading edge of tumor will exhibit phenotypes that can pioneer in a novel, and sometimes hostile environment. Interestingly, this prediction is consistent with observations in nature that "weedy" phenotypes (i.e. higher maximum proliferation rates at the expense of lower carrying capacities) predominate at the leading edge of a population invasion when compared to individuals in regions far from the propagating border.

Clinical Analysis

As described above, the evolutionary models predict observable changes in the neoplastic cells and the environment in both the center and edge regions of a tumor. We tested these model predictions with clinical analysis of histological sections evaluated by quantitative image analysis. Our clinical results indicate a number of consistently observed and quantified changes in cell density, cell proliferation, cell death, cell aggression, acidosis, and hypoxia in both locations (each measured in triplicate) of histological samples of ten invasive breast cancer patients. Furthermore, the presence of lymphocytes and vascular resources in the microenvironment were measured in triplicate in both locations of the same tumors (See Table 2.1; Table 2.2; Figure 2.4).

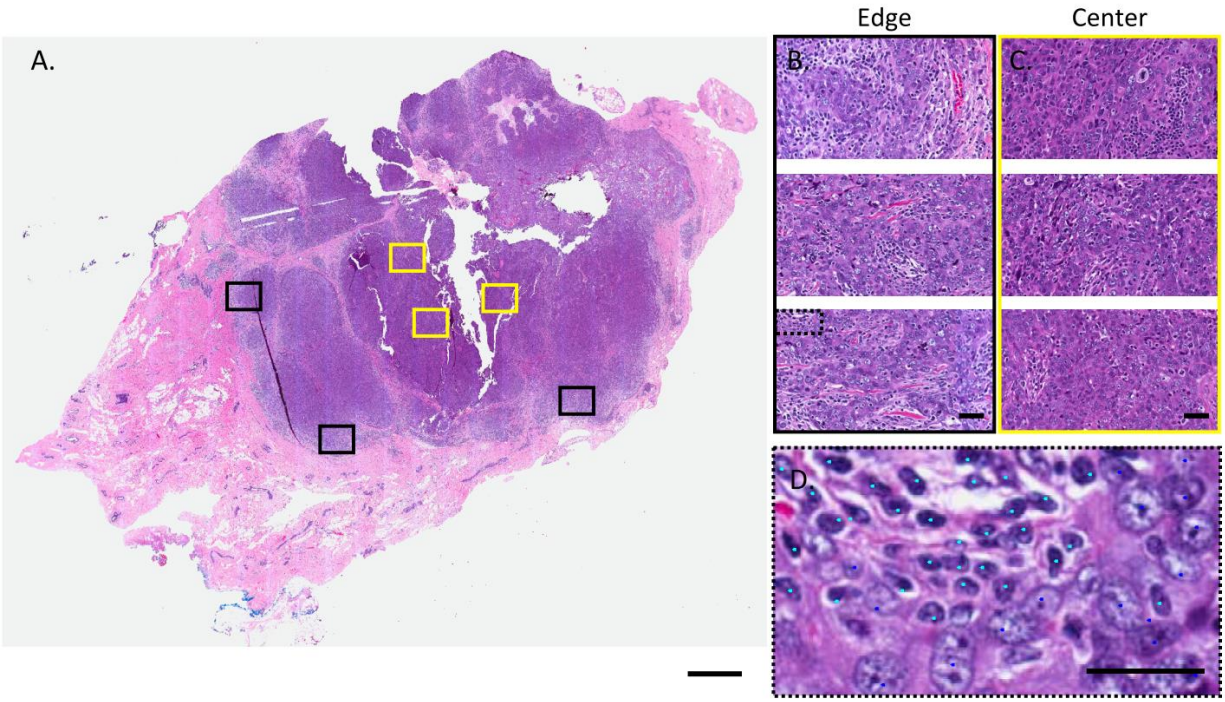

Figure 2.4 H&E images of a Grade III invasive breast cancer. A) Regions were randomly selected from the whole slide image such that three regions were within 1mm of the edge of the tumor border (black boxes) and three regions were located near the center of the tumor region (yellow boxes). Scale=2mm. B) Each edge region and C) each center region are shown at 200x magnification. Scale =100µm. D) is a digitally zoomed to 1000x from the dotted black box and demonstrates the tumor cell identification (blue points) and lymphocyte identification (teal points) used to calculate both the tumor cell density and lymphocyte numbers in each of the 60 H&E and 600 total images evaluated. Scale =100µm.

Metric	Unit	Center	Edge	C:E	
Cell Density	Avg #/ Area	594	503	1.18	0.5413
CA9 Expression	% Strong +	5	19	0.24	0.1953
CA12 Expressio	% Strong +	40	11	3.65	0.785
KI67	Avg #/ Area	78	190	0.41	0.2901
CC3	Avg #/ Area	26	11	2.42	0.7074
Glut1	% Strong +	6	20	0.29	0.225
Hif1α	% Strong +	19	14	1.35	0.5751
CD34	Avg #/ Area	1	2	0.38	0.2735
Lymphocytes	Avg #/ Area	87	89	0.97	0.4919

Table 2.1 Summary of the biomarker measurements comparing center and edge in 10 patient samples.

		Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10
Cell Density	Center ROI 1	857	672	589	614	609	387	561	516	454	662
# Tumor Cells	Center ROI 2	836	606	532	542	387	349	782	494	488	814
	Center ROI 3	812	543	607	665	669	507	530	612	527	586
	Edge ROI 1	717	614	487	568	396	299	492	492	630	611
	Edge ROI 2	664	549	416	586	368	314	500	420	417	666
	Edge ROI 3	799	278	443	316	465	394	500	518	457	714
CAIX	Center ROI 1	1.641	4.716	NA	1.387	4.738	3.814	6.519	2.314	8.517	4.62
% Positive	Center ROI 2	3.773	2.874	NA	2.401	5.531	6.558	8.494	4.666	11.068	2.873
	Center ROI 3	1.264	5.924	NA	1.433	5.49	4.962	4.743	0.129	9.083	2.113
	Edge ROI 1	12.244	23.966	NA	6.1	21.003	14.478	14.538	11.078	16.369	30.157
	Edge ROI 2	16.105	24.302	NA	17.819	21.578	23.937	17.19	9.702	26.057	29.387
	Edge ROI 3	17.247	27.922	NA	11.247	11.598	10.846	18.455	13.244	27.433	27.25
CAXII	Center ROI 1	44.128	26.53	NA	49.732	29.437	46.278	36.328	33.508	38.324	18.08
% Positive	Center ROI 2	47.199	26.284	NA	36.973	39.067	52.71	55.579	45.049	54.482	22.479
	Center ROI 3	40.314	26.636	NA	63.171	28.822	52.982	43.371	45.473	48.946	27.266
	Edge ROI 1	19.386	6.26	NA	12.007	3.535	25.121	2.415	8.8	28.132	0.947
	Edge ROI 2	22.303	12.511	NA	12.899	4.696	11.153	5.044	2.927	27.131	0.275
	Edge ROI 3	27.733	7.815	NA	12.735	2.096	5.301	6.821	4.764	21.544	1.298
Ki67	Center ROI 1	110	121	54	23	78	18	75	14	25	150
# Positive Cells	Center ROI 2	175	115	51	71	80	31	57	10	16	238
	Center ROI 3	140	119	48	19	69	23	56	12	31	298
	Edge ROI 1	402	484	86	67	104	84	138	17	157	390
	Edge ROI 2	294	340	147	101	89	146	227	59	147	427
	Edge ROI 3	229	190	107	63	115	116	197	161	104	507
CC3	Center ROI 1	7	25	43	19	29	27	57	2	11	102
# Positive Cells	Center ROI 2	16	14	23	16	3	31	37	11	17	78
	Center ROI 3	21	17	18	16	12	11	34	9	23	59
	Edge ROI 1	6	7	21	4	8	10	4	7	4	24
	Edge ROI 2	8	6	7	24	11	18	11	5	8	12
	Edge ROI 3	4	11	18	8	6	19	2	9	13	31
Glut1	Center ROI 1	0.314	8.471	1.29	6.56	3.258	4.933	4.93	0.854	32.024	4.524
% Positive	Center ROI 2	0.129	5.811	0.747	6.287	1.021	6.131	5.284	0.319	15.637	5.445
	Center ROI 3	0.187	10.771	0.348	4.792	0.368	4.672	7.931	0.4	23.765	2.745
	Edge ROI 1	0.318	39.921	24.749	5.828	1.419	40.06	36.891	25.447	10.046	18.956
	Edge ROI 2	3.67	42.224	6.458	8.177	3.993	41.144	29.27	12.701	6.928	20.507
	Edge ROI 3	1.521	38.999	12.13	2.501	7.489	51.689	31.168	27.471	1.359	32.348
HIF-1α	Center ROI 1	10.964	12.786	2.569	10.062	15.997	29.895	20.92	11.241	26.848	36.612
% Positive	Center ROI 2	8.603	33.624	3.353	7.354	19.413	19.633	34.197	8.18	28.975	37.814
	Center ROI 3	14.684	14.923	3.396	5.759	22.511	30.358	11.925	8.244	27.183	41.661
	Edge ROI 1	7.176	12.218	2.084	6.036	16.464	16.219	5.618	5.791	26.769	39.353
	Edge ROI 2	6.452	8.331	0.736	11.41	19.223	34.368	4.054	6.341	18.317	31.876
	Edge ROI 3	9.817	15.429	1.32	6.834	16.234	12.809	4.144	9.244	20.748	38.165
CD34	Center ROI 1	0.454	0	0.353	1.78	0.483	0.11	0.728	6.007	0.191	0.031
Vascular Area	Center ROI 2	0.516	0.091	0.49	0.706	0.431	0.211	0.345	4.376	0.534	0.018
	Center ROI 3	0.676	0.104	0.565	0.761	0.544	0.178	0.768	2.227	0.193	0.236
	Edge ROI 1	0.969	0.689	1.783	1.374	1.269	1.502	1.055	3.465	1.626	0.608
	Edge ROI 2	1.2	1.285	5.357	4.725	1.091	0.215	1.623	9.114	1.66	1.025
	Edge ROI 3	0.535	0.528	2.521	1.751	1.307	0.55	1.246	12.148	0.82	0.996
Lymphocytes	Center ROI 1	268	30	57	75	212	30	48	65	15	118
# Lymphocytes	Center ROI 2	183	29	29	111	115	50	40	124	33	84
	Center ROI 3	262	26	104	202	60	24	81	46	7	70
	Edge ROI 1	245	71	5	4	12	58	42	172	67	56
	Edge ROI 2	282	23	12	55	108	25	90	93	28	228
	Edge ROI 3	355	11	22	11	138	21	66	87	27	270

Table 2.2 Comprehensive data of the biomarker measurements comparing center and edge in 10 patient samples (each measured in triplicate).

First, the tumor cell density was evaluated. We used a partially-hierarchical ANOVA (SYSTAT v13) to test for the effects of tumor cell density (number of tumor cells per area) and habitat (tumor center versus tumor edge; each with triplicate sampling) for each of 10 patients. These analyses were calculated by quantifying the number events (as indicated as the number of tumor cells or, for each biomarker, strongly expressing tumor cells) within a 500µm by 500µm subregion. Each subregion was extracted randomly, in triplicate, from within 1mm of the mesenchymal interface for edge samples and beyond 1mm from the mesenchymal interface for center regions. The cell density model provided a good fit to the data (multiple r2=0.72). The patient (F-Ratio=0.49, not significant [n.s.]) was not found to influence the ratio of cell counts per region so that the tissue slices provided roughly the same ratio of cancer cells regardless of patient. Cancer cell abundances varied significantly by center and edge region (F-Ratio=15.39, P<0.001) for all patients (F-Ratio=9.20, P<0.001). This indicates that cells in the tumor center out-numbered cells at the tumor edge consistently across all patients with statistical significance.

Second, the tumor cell proliferation was evaluated by evidence of Ki-67 expression across tumor cells in triplicate for both regions for the same 10 patients. The model also provided a good fit to the data (multiple r2=0.88). Again the patient (F-Ratio=2.74, not significant [n.s.]) was not found to influence the ratio of Ki67 positive cells per region so that the tissue slices provided roughly the same ratio of Ki67 positivity regardless of patient. Proliferation varied very significantly by center and edge region (F-Ratio=73.58, P<0.001) for all patients (F-Ratio=21.56, P<0.001). This indicates that proliferative cells in the tumor edge consistently out-numbered cells in the tumor center across all patients with statistical significance.

Next, the tumor cell death by apoptosis was evaluated by cleaved caspase 3 (CC3) expression across tumor cells in triplicate for both regions for the same 10 patients. The model also provided a good fit to the data (multiple r2=0.84). Here CC3 expression varied very significantly by center and edge region (F-Ratio=45.23, P<0.001) for all patients (F-Ratio=12.04, P<0.001). This indicates that apoptosis in the tumor center consistently out-numbered cells in the tumor edge across all patients with statistical significance.

Then, a number of additional metabolomic biomarkers which indicate tumor cell aggression, acidosis, glycolysis and hypoxia were tested. Aggressive, acid producing cells should be consistently observed in the tumor edge while cells in vascularized regions of the remainder of the tumor should be functioning in normal pHe. To test model predictions, we examined the spatial distribution of Carbonic Anhydrase IX and XII (CAIX and XII) as biomarkers for regional high and low acidity respectively.

The model provided a good fit to the CAIX data (multiple r2=0.91) and the CAXII data (multiple r2=0.93). CAIX expression varied very significantly by center and edge region (F-Ratio=255.77, P<0.001) for all patients (F-Ratio=7.88, P<0.001) whereas CAXII expression varied very significantly by center and edge region (F-Ratio=329.27, P<0.001) for all patients (F-Ratio=12.50, P<0.001). This indicates that CAIX expressing cells in the tumor edge consistently out-numbered cells in the tumor center across all patients with statistical significance when the converse is true of CAXII which consistently has higher expression in the tumor center across all patients with statistical significance. This matched with the predictions of the evolutionary mathematical models (Figure 2.5).

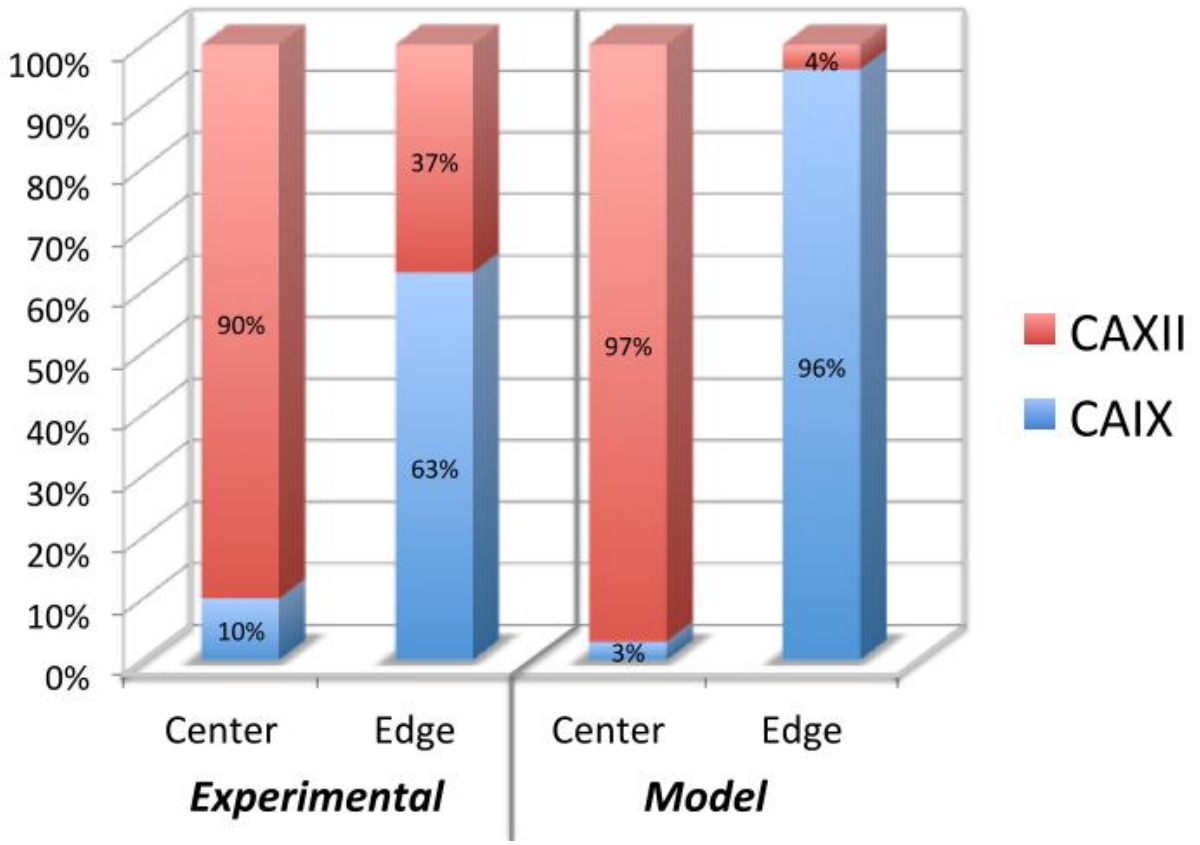

Figure 2.5 Experimental and mathematical model results showing the percentage of total cells counted in the center and edge that expressed either CAIX or CAXII. Experimental results showed that 90% of the cells in the center of the tumors expressed CAXII while only 10% expressed CAIX. Conversely 63% of the cells in at the edge of the tumors expressed CAIX. The mathematical model showed that 97% of the cells in the center would express CAXII and 96% of the cells at the edge would express CAIX.

Glycose transporter 1 (GLUT1), also known as solute carrier family 2 facilitated glucose transporter member 1 (SLC2A1) (multiple r2=0.93) and hypoxia-inducible factor 1-alpha (HIF1α) (multiple r2=0.86) measurements were similarly modeled. GLUT1 expression varied very significantly by center and edge region (F-Ratio=148.70, P<0.001) for all patients (F-Ratio=22.08, P<0.001) whereas HIF1α expression varied very significantly by center and edge region (F-Ratio=13.40, P=0.001) for all patients (F-Ratio=24.44, P<0.001). This indicates that GLUT1 expressing cells in the tumor edge consistently out-numbered cells in the tumor center across all patients with statistical significance when the converse is true of HIF1α which consistently has higher expression in the tumor center across all patients with statistical significance.

Finally, two aspects of the environment were quantified- the vascular density (number and area of vasculature) and the density of lymphocytes per region area. The number of blood vessels per area (multiple r2=0.80), the area of vascular involvement (multiple r2=0.77) and the density of lymphocytes (multiple r2=0.79) were modeled. The number of blood vessel per area varied significantly by center and edge region (F-Ratio=22.48, P<0.001) for all patients (F-Ratio=12.66, P<0.001) whereas the area of vascular involvement varied significantly by center and edge region (F-Ratio=16.85, P<0.001) for all patients (F-Ratio=11.62, P<0.001) whereas lymphocytic density did not vary significantly by center and edge region (F-Ratio=0.057, n.s.) but was consistent for all patients (F-Ratio=14.71, P<0.001). This indicates that the number and area of vasculature was statistically greater at the tumor edge than in the tumor center across all patients however the lymphocytes were statistically similar in both the edge and center regions across all patients. Non-specific staining of one of the patients may have resulted in an artificially high patient interaction effect. When this patient's samples were removed the trend remained constant for the center to edge effect (F-Ratio=27.25, P<0.001) and the patient effect was reduced to (F-Ratio=2.89, n.s.). The lymphocytic response, taken together with the tumor cell density does however indicate that the ratio of lymphocytes to tumor cells is consistently high at the tumor edge (where the tumor cell density is lower than the center).

Overall, the biomarker measures of tumor cells are strikingly segregated by center and edge regions. This species by habitat interaction contributes most to the explained variation within the statistical model (Table 2.3). See Figure 2.6 for a graphical representation of each data point.

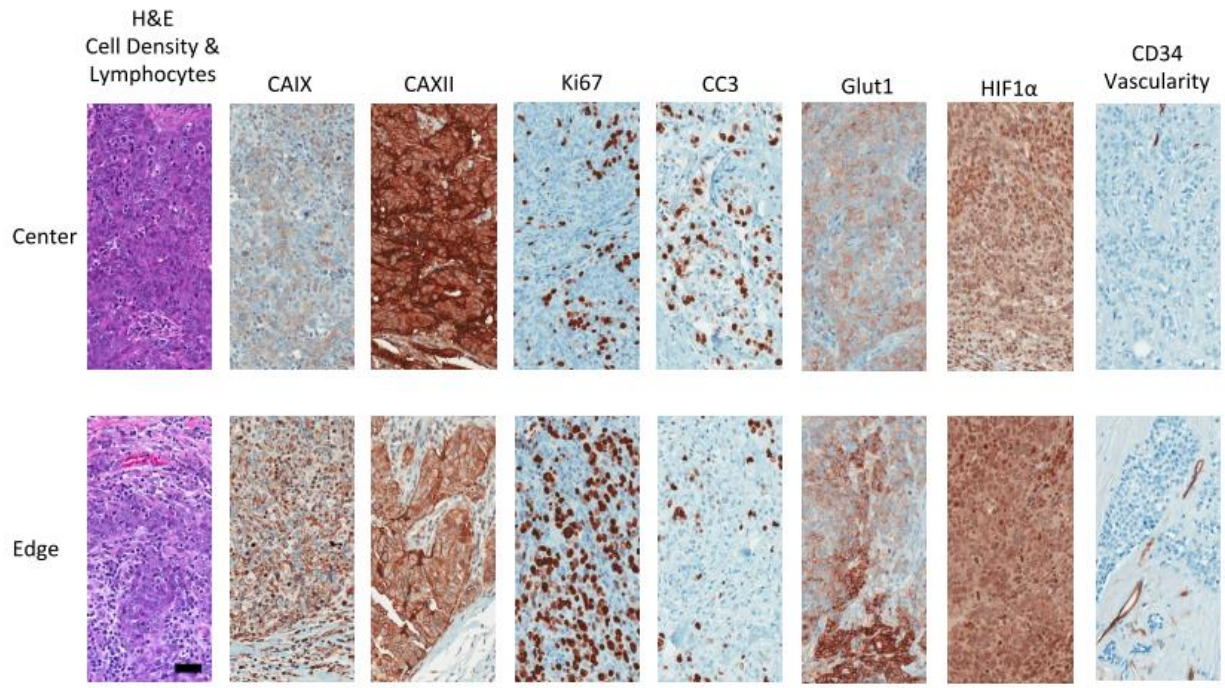

Figure 2.6 A) An image panel of center (top) and; B) edge (bottom) regions are displayed to demonstrate examples of each biomarker staining within each area of interest. Scale =100µm.

	multiple r^2	F-Ratio (habitats)	F-Ratio (patients)	F-Ratio (habitats*patients)	p-Value (habitats)	p-Value (patients)	p-Value (habitatis*patients)
Cell Density	0.072	15.387	9.297	0.495	<0.0001	<0.0001	n.s.
CAIX	0,908	255.766	7.881	4.513	<0.0001	<0.0001	0.001
CAXII	0.927	329.297	12.495	3.318	<0.0001	<0.0001	0.006
Ki67	0.88	73.58	21.559	1.516	<0.0001	<0.0001	n.s.
CC3	0.838	45.231	12.036	5.993	<0.0001	<0.0001	<0.0001
Glut1	0.933	148.704	22.081	1.692	<0.0001	<0.0001	n.s.
HIF-1α	0.861	13.4	24.44	1.516	0.001	0.001	n.s.
CD34 (#)	0.797	22.482	12.661	2.261	<0.0001	<0.0001	n.s.
CD34 (#)	0.64	27.25	2.886	1.692	<0.0001	0.014	n.s.
Lymphocytes	0.793	0.057	14.708	2.744	0.812	<0.0001	n.s.

Table 2.3 Statistical summary for each biomarker by region interaction.

Table 2.3 Statistical summary for each biomarker by region interaction.

Discussion

The conventional model intratumoral evolution allows new “driver” mutations to accumulate indefinitely thus implicitly assuming that tumor cells never achieve a local fitness maximum. Here we explore an alternative model in which cancer cells rapidly evolve to an evolutionary stable state (ESS) and, thus, cannot be displaced by new strategies if the environment remains stable. This would lead to local phenotypic convergences so that regional molecular variations, rather than the result of random mutations, would represent reasonably predictable phenotypic adaptations to changes in environment conditions such as blood flow that are potentially observable through conventional clinical imaging such as MRI. This model would be supported by identification of a simple, consistent spatial variation in tumor molecular properties that emerged directly from fundamental evolutionary dynamics

Here, we framed this hypothesis mathematically using evolutionary game theory. Although built on a simple conceptual model, computer simulations demonstrate the ecological dynamics within cancers can be quite complex and highly variable from tumor to tumor. However, as noted above one common pattern emerged. Consistent with the spatial patterns observed in species invasions in nature, our models predicted the tumor cells at the invasive front of the tumor will possess distinct phenotypic properties when compared to the cells in the core.

To test the results of the model described above, we performed a detailed analysis of spatial molecular heterogeneity in ten clinical breast cancers demonstrates a consistent regional distribution in which proliferation, the ratio of tumor cells to lymphocytes, GLUT1 and CAIX expression were higher at the tumor edge. Conversely, tumor cell density, apoptosis, HIF1α and CAXII expression were observed to be greater in the tumor center. While the number of clinical tumors is small, we note that the results are highly statistically significant. Furthermore, other clinical studies have observed changes in gene expression in the edge of cutaneous squamous cell carcinoma [26, 27] and colon cancer [28].

Our results are similar to the variations in favorable and unfavorable gene signatures within the same tumor reported in prior studies [1,3]. For example, our results show that positive prognostic (CAXII) and negative prognostic (CAIX) biomarkers are routinely observed in the same tumor but different regions. Importantly, however, we can clearly identify the Darwinian dynamics that produced this spatial variation and thus place this regional heterogeneity within a predictable evolutionary process. This has important clinical implications because it supports the hypothesis that intratumoral heterogeneity is significantly influenced by environmental variations such as blood flow.

Our results suggest a number of important avenues for future investigation. Since clinical cancer imaging can depict spatial variations in perfusion, it should be possible to estimate some molecular variations based on imaging. In addition, it seems clear that some current prognostic and predictive molecular biomarkers that can be observed in different regions of the same tumor, such as CAIX and CAXII, can be accurately evaluated and reported only in a spatial context.

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III. PATHOLOGY TO ENHANCE PRECISION MEDICINE IN ONCOLOGY: LESSONS FROM LANDSCAPE ECOLOGY

Abstract

A major goal of modern medicine is increasing patient-specificity so that the right treatment is administered to the right patient at the right time with the right dose. While current cancer studies have largely focused on identification of genetic or epigenetic properties of tumor cells, emerging evidence has clearly demonstrated substantial genetic heterogeneity between tumors in the same patient and within subclones of a single tumor. Thus, molecular analysis from populations of cells (either a whole tumor or small biopsy of that tumor) is, at best, an incomplete representation of the underlying biology. These observations indicate a significant need to define intratumoral evolutionary dynamics that yield the observed spatial variations in cellular properties.

It is generally accepted that genetic heterogeneity among cancer cells is a manifestation of intratumoral evolution, and this is typically viewed as a consequence of random mutations generated by genomic instability within the cancer cells. We suggest that this represents an incomplete view of Darwinian dynamics, which typically are governed by phenotypic variations in response to spatial and temporal heterogeneity in environmental selection forces. We propose that pathologic feature analysis can provide precise information regarding regional variations in environmental selection forces and phenotypic adaptations. These observations can be integrated using quantitative, spatially-explicit methods developed in landscape ecology to interrogate heterogeneous biological processes in tumors within individual patients. The ability to investigate tumor heterogeneity has been shown to inform physicians regarding critical aspects of cancer progression including invasion, metastasis, drug resistance and disease relapse.

Introduction

Personalized medicine aims to use patient-specific metrics to provide an optimal cancer therapy customized for each individual patient (Maitland 2006; Howland 2012; Mirnezami 2012). Massive biobanks of patient tissues provide extensive libraries of genetic data that can be evaluated against targeted therapies (de Souze 2012). However, it is becoming clear that discriminating and cataloguing genomic libraries of patient samples falls short, due in part to intratumoral heterogeneity. Personalized cancer treatments will require more than just matching a patient's tumor genomics with that of a central library.

Detailed molecular data from multiple regions in the same tumor reveal striking variations. Distinct populations of tumor cells displaying different biomarkers and gene signatures appear to co-exist (Gerlinger 2012). This invites a greater understanding of tumor heterogeneity at molecular, cellular, and tissue temporal and spatial scales (Powathil 2012; Faratian 2011). Unfortunately, current proteomic and genomic methods fail to wholly address heterogeneity. Current techniques rely on single sample that homogenizes into large numbers of undoubtedly variable cells. It is likely that even these "averaged" data will differ from region to region within the same tumor, and certainly between tumor sites in the same patient (Rabes 1979; Askensten 1989; Raychaudhuri 2012). Batching and averaging information from millions of cells is likely limiting for developing personalized cancer treatments.

We propose extending pathology to identify, classify and quantify cell to cell, region to region, and tumor to tumor heterogeneities. Such pathology metrics can supplement current efforts towards personalized medicine. We propose analyzing histological samples by employing the theories, tools and experiences of landscape ecology.

Landscape ecology measures, analyzes and studies the spatial and temporal heterogeneities of natural ecosystems (Turner 1989). Since the pioneering work of Carl Troll in 1939, landscape ecologists have used maps, vegetation and geologic surveys, photographic images and, most recently, satellite imaging to study the interactions between organisms with their environments. While maps are not the only tools of

landscape ecology, these data acquisition methods empower investigators to study spatially explicit biological interactions. Together with information about organisms and the patterns of the organism's environment, investigators can interrogate habitat change, conservation and other ecological interactions. We propose that many of these same principles and techniques can be developed and applied to create an emerging field of "landscape pathology". While there are many definitions of 'landscape', we are using the definition of landscape from Turner, Gardner, and O'Neill (2001) which states that "a landscape is an area that is spatially heterogeneous in at least one factor of interest". Thus, we define landscape pathology as a proposed discipline to apply quantitative, spatially-explicit methods from landscape ecology to define the heterogeneous biological processes of cancer cells (the 'organism') in histological samples (the 'habitat'). Using landscape pathology methods can help investigators gain a more precise understanding of local selection forces and in turn, adaptations within subpopulations of cancer cells in a tumor, which may be clinically important in understanding disease progression, treatment response and relapse.

Figure 1A and B provides an example of this concept. Here, hematoxylin and eosin (H&E) stained histology slides, the standard for primary diagnoses in cancer, may be compared with traditional landscapes commonly viewed as maps or satellite imagery. Regional classifications, spatially-explicit analyses and quantifiable metrics common in ecology can add to the pathologist's clinical toolbox (Park 2012, Sexton 1990). These classifications and analyses will provide pathologists with the ability to quantify morphological heterogeneity within a tissue section with exquisite precision. This in turn, will empower pathologists with the ability to rapidly identify areas of necrosis, high rates of proliferation, high incidences of inflammatory response, regions of high or low nuclear pleomorphism and similar clinically pertinent morphological features.

The Clinical Problem

Intratumoral heterogeneity manifests in at least three general ways:

1. Mixtures of normal and malignant cellular populations within tissue. Pathologists typically describe this variation in qualitative terms. The pathologists will, for example, recognize benign cells such as fibroblasts,

lymphocytic and epithelial cells. They will also identify inflammation, necrosis, hyperplasia, pre-neoplastic disease, benign tumors and malignant cancers [Edge 2010].

2. Variations in the microenvironment. Blood flow in tumors typically results in temporal and spatial variations in concentrations of growth factors, substrate, and metabolite concentrations. These in turn manifest as regions of necrosis and variable cell density. Each of these variations selects for the adaptive evolution of local tumor populations which can be spatially evaluated in a quantitative manner including, but not limited to spatial evaluation of clustering populations or proliferative phenotypes [Potten 1982].

3. Genetic heterogeneity. Increased mutation rate owing to intracellular properties such as DNA repair or genotoxic environmental factors such as hypoxia appear to continuously generate new mutant cells. Cells carrying these mutations, in turn, proliferate if the phenotypic expression of that mutation confers an increased fitness. An example is the perpetuation of the mutator phenotype [Loeb 1991].

In current practice, pathologists attempt to overcome this heterogeneity by selecting regions of tissues for genetic analysis that minimizes normal or necrotic cells. This is commonly performed using a slide marker to draw directly on the glass slide in an effort to select regions of high tumor cellularity or conversely, to scratch out large regions which are not of interest including normal margins or necrosis. In this way the technique currently “homogenizes” the samples and averages any downstream information which may be useful in evaluating intratumoral heterogeneity [Blin 1978, Kristo 2013].

We propose that current methods for personalized cancer therapy--treating target lesions as a single heterogeneous genetic sample--are not wholly adequate. This is primarily because evolutionary strategies or adaptations often involve several phenotypic changes which in turn can be achieved through an even larger number of genetic pathways. That is, evolution directly acts on cellular phenotypes and not

genotypes. In fact, Darwinian dynamics, which can be described as dynamics of systems which drive fitness by natural selection, are manifested in phenotypic changes (Yuan 2012). Second, clear evidence indicates that extensive genetic heterogeneity exists within cancer cells in the same tumor (Gerlinger 2012). Averaging or lumping tumor heterogeneity into single metrics or qualities may mask key aspects of the tumor's progression and state, and unwittingly "throw away" valuable information on the heterogeneity itself and what it indicates (Merlo 2006). We suggest embracing the information content of spatially-explicit considerations of cellular, microenvironmental and regional heterogeneities. The ability to investigate tumor heterogeneity has been shown to inform physicians regarding critical aspects of cancer progression including invasion, metastasis, drug resistance and disease relapse (Fidler 1978; Marusyk 2010; Heppner 1983; Dexter 1986).

It is important to understand why intratumoral heterogeneity is clinically important. Clinicians have deep experience with disease relapse, changes in treatment effectiveness and hormone status or other clinically relevant deviations in the greater cancer cell population. We propose that the described changes in the patient's overall disease state are due in significant part to cancer cell populations changing as different sub-populations of cancer cells evolve towards increased fitness by natural selection. Intratumoral heterogeneous subpopulations of different cellular phenotypes in a single tumor make these Darwinian dynamics possible, which in turn makes treating cancer a moving target.

Cancers are complex but not hopelessly so. Tumors can be understood by characterizing and embracing their underlying ecological and evolutionary dynamics, including both spatial and temporal variability. Fortunately, pathologists have been observing and quantifying variations within the morphology of cancer cells for well over a century (Wilks 1874) and are well positioned to visually evaluate heterogeneity across histological samples. We propose that the challenges are threefold: Identification of cellular heterogeneity must become 1. Regionally explicit 2. Quantitative and reproducible and 3. High throughput.

Until recently, these challenges would be insurmountable due to the time consuming and subjective manual screening of tumor heterogeneity (Campbell 2007). However, with the advent of whole slide imaging technology and recent improvements in pattern recognition software it is possible to computationally evaluate millions of cells in minutes and hundreds of patients in hours to days (Chantrain 2003). Whole slide imaging allows high resolution and high throughput image acquisition for every cell within a given tissue sample (Romer 2003). There are a number of advantages of these technologies including low costs, high throughputs, quantitative results and rapid evaluation of tissue samples which are already routinely produced in every hospital within the United States and the majority of similar centers around the world (Park 2012).

While current imaging technology and methods are necessary, it is not sufficient to visually investigate spatial distribution of cells and microenvironmental properties in patient samples. We propose that the discipline of landscape ecology, with its associated tools and theories, can be used to evaluate the relationship between pattern and process in pathology. While pathology is equipped to identify patterns in tumors, landscape ecology provides the tools to evaluate these patterns and understand the underlying biological relationships (Addicott 1987; Tischendorf 2001; Diaz 2012). Together, quantifiable metrics of digital pathology and landscape ecology can contribute to personalized medicine. Thus, "landscape pathology" has the potential to provide new information regarding patients' intratumoral heterogeneity.

Benefits of Landscape Ecology in Pathology

Landscape ecologists and pathologists work on a spectrum of scales which spans from 106 meters to 10-6 meters, yet the substrates on which both groups work is identical. In modern studies, these are most frequently digital images, whether from a satellite or a microscope lens. Whole slide microscope images are the maps with which landscape pathologists will work. Distinctive heterogeneous regions can be identified along with their relative contribution to the total tumor volume as well as their interactions with each other. This is done by using segmentation and classification methods (Sexton 1990) to identify distinct regions in the tumor and to allow examination of their boundaries.

The first consideration for segmentation and classification in tumors may be the criteria with which regions will be identified. In landscape ecology, “patches of the land cover region” can be wetlands, clusters of trees, or any relatively homogeneous area of interest. Habitat patches are identified in a number of different ways including reflectance data, time series of vegetation activity estimated from the reflectance data, estimates of surface roughness/vertical structure from RADAR or LiDAR sensors or surface temperature from thermal remote sensing, to name a few (Kerr 2003; Goetz 2010; Andrew 2009). These variables, which a landscape ecologist may use, might then be chosen to classify habitat using a number of pixel or object based methods (Gustafson 1998; Jobin 2008). Pixel and object based classification methods are also common in the current standards of digital pathology analysis (Naik 2008; Teverovskiy 2008). In fact, pathologists now have a plethora of commercially available image analysis tools to segment objects (i.e. delineate patches) and classify spatially explicit regions (i.e. identify habitats) by examining vascular density, relative cell viability and necrosis or immunohistochemical evidence of regional oxygen concentration such as HIF-1α expression (Tansey 2004).

As demonstrated in Figure 1 C and D, histological pattern recognition utilizes random forest classification to identify color and texture variation in both image types via a commercially available image analysis platform (Definiens, Munich, Germany). By teaching the algorithm to recognize homogeneous regions, a computer can reliably identify “patches” in a robust and repeatable way. Individual physical or molecular features derived from the images can be used to catalog morphologically homogeneous cell populations and relationships between cells or regions and establish testable hypotheses to further interrogate processes of the system (Singh 2011). Similar to the patch-matrix (also sometimes referred to as patch mosaic) paradigm in landscape ecology practices, histological pattern recognition also creates and thus defines a border between tissue regions (Pickett 1995). Of course, alternatives in landscape ecology to evaluate spatial heterogeneity such as gradient paradigms are also used (Cushman 2010).

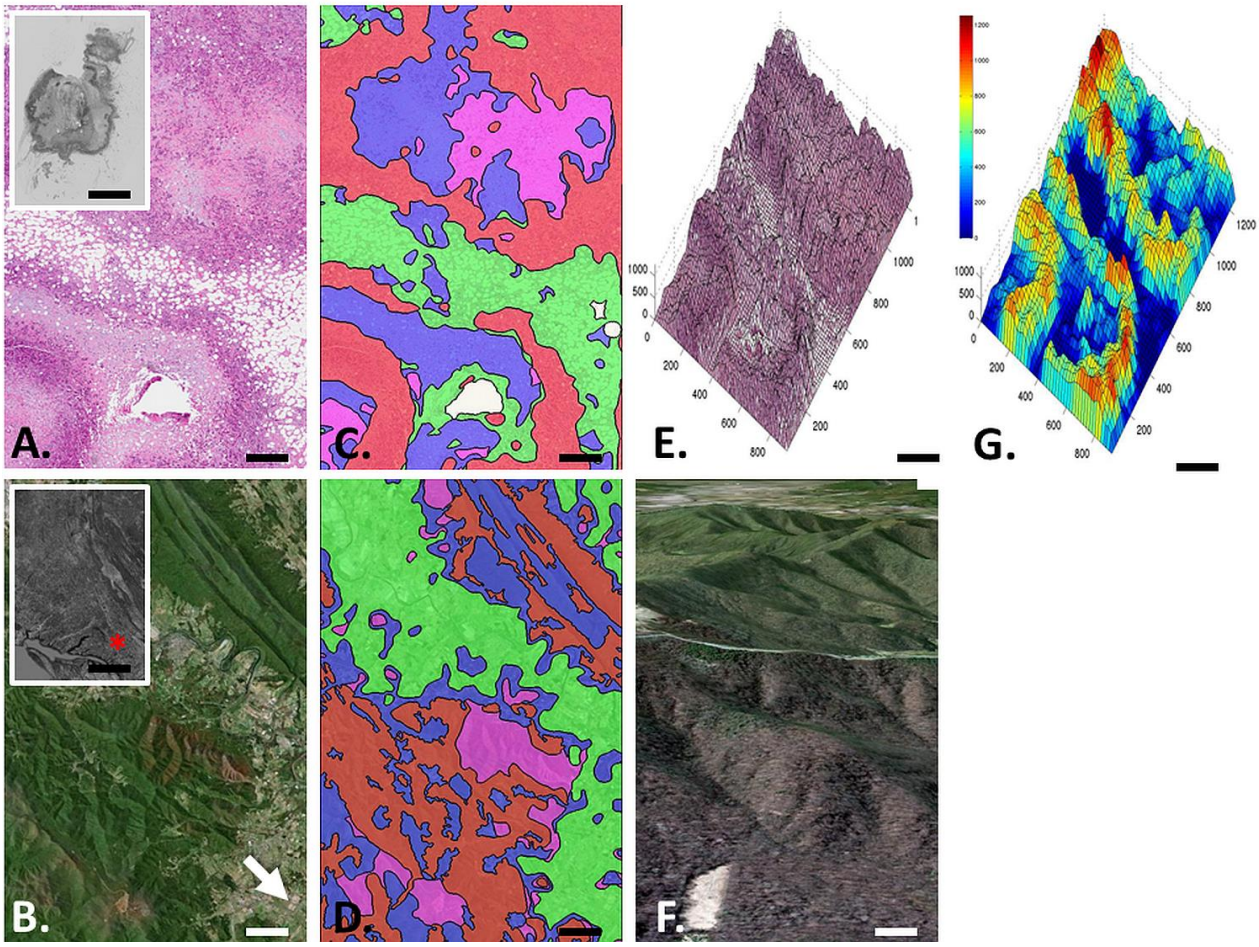

Figure 3.1 Hematoxylin and eosin (H&E) stained histology slides may be compared with traditional landscapes. A) A standard histological H&E image of an invasive tumor compared to B) a standard satellite image of the Blue Ridge mountain range in Shenandoah, Virginia. C) Regional tissue classification of viable tumor (red), mild necrosis (purple), necrosis (blue) and other non-target tissue (green); D) compared to random forest classification in variant habitats which seems to correlate most with increasing elevation from green to blue to red to purple. E) Feature data is displayed in the Z dimension to create a topographical map of the H&E relative to the morphological feature being evaluated (red: blue color layer ratio); F) 3D topographic representation of the Shenandoah habitat site demonstrating heterogeneous elevation. G) Topographical heatmap is used to rapidly identify regions with like feature characteristics for this H&E image. Top scale bars (A, C, E, G) represent 300µm and the top insert map scale bar is 3mm; bottom scale bars (B, D, F) represent 3000m and the bottom insert scale bar is 100km. Credit Google Earth for all satellite imagery. The white arrow indicates North. The red asterisk is the location of Washington D.C., USA.

Qualitatively, pathologists could then begin to evaluate the regions of interest with these or other classification methods of patches. With quantitative image analysis and statistical methods, pathologists can quantify precise metrics of the regions (Sparks 2010). Initially these metrics might include area, intensity, roundness and other physical features (Lloyd 2010). However pathology can learn from

landscape ecology that patches can form mosaics (Ludwig 2000). They can demonstrate edge effects and they can be clustered (Cottam 1956; Fraterrigo 2005). These features of tissue architecture can also be quantitatively measured and used to evaluate the disease at a mesoscopic scale (200µm-2mm). For example, the architecture of prostate glands and their orientation have been shown to be informative of patient prognosis (Madabhushi 2011) and retained architecture in breast cancer tissues has been shown to inhibit of malignant progression (Bissel 2010).

Multiple regions or patches typically exist in a landscape. We expect that similar variations will be found in most clinical cancers using the tools for landscape pathology (Watt 1947; Dorner 2002). In turn, this will allow the intratumoral heterogeneity to be characterized, quantified and ultimately compared. A way in which the heterogeneity may be characterized can include habitats of vascular regions (Gatenby 2013; Alfarouk 2013 and Lloyd 2014) or regions of increased lymphocytic response (Messina 2012). Each quantitative method cited includes clinically meaningful prognostic or predictive value.

Landscape Ecology Applications in Pathology

Evaluation of the consequences of having multiple subpopulations of cells in specific patterns in a tumor will require learning from landscape ecology, which focuses on the feedback loops between patterns and processes. Patterns observed in pathology include points of blood vessels or ectopic lymph nodes, expression levels of biomarkers like Her2 or regions of high proliferation. Each of these patterns has diagnostic and prognostic value to the pathologist. Here we describe four analyses and their utility to pathology.

The first is point patterns, which consist of point locations distributed in two-dimensional space. Landscape ecologists would characterize these as random, regular or clustered. In pathology, point pattern analysis would allow quantification of the spatial distribution of some cellular or tissue feature (i.e. nuclei) in the tumor. Metrics such as Ripley's K function (Wiengand 2004; Haase 1995) can be used to compare one region of the tumor to another. Specifically, Ripley's K is a statistical metric for quantifying deviations from

spatial randomness and has been used in mammography as a classification method (de Oliveria Martins 2007). Point patterns in a histological section have been shown to be important at the histological scale in interrogating aspects of the environment including lymphocytic invasion, cancer associated fibroblastic localization, or the distance from vasculature to cancer cell populations (Thomlinson 1955, Kim 2001). This information could help oncologists most carefully predict response to specific therapies.

Second, regional variations in necrosis, ectopic lymph nodes or other intratumoral features can be described as values of the number of cells, size of necrotic regions or distribution of lymphocytes in space. In this example, measures of spatial autocorrelation, such as Moran's I (Moran 1950; Rocha 2014) can reveal the scale and degree of dependency among observations. This is, for example, important to be useful to quantify dispersal (migration) of specific cell populations by evaluating the pH of the microenvironment (Estrella 2012). Specifically, the location of cancer cells can be quantified to better understand if cancer cells are moving together in regions in which acid-mediated invasion provides spaces of increased selective advantage or if invasion is more correlated with random Brownian motion.

These metrics may also be useful in predicting response to specific treatments like hypoxia activated pro-drugs or predicting prognosis of the patient's own immune response (Messina 2012). Specifically, the spatial correlation of cancer cell populations is useful in understanding the overall heterogeneous organization of cancer cells. That is to say, cancer is not randomly oriented or unorganized, but rather is a function of selective Darwinian dynamics of an adaptive landscape which may be measured and investigated. Furthermore, quantification of spatial relationships among tumor cell clusters of networked populations in relation to these environmental responses can be used to reveal prognostic indicators such as increased nuclear pleomorphism in regions of increased vascularity (Delahunt 1997; Jellinger 1975).

Thirdly, distance measures are frequently used tools to collect quantitative data in histological images (González-García 2012). For example, number of interactions with neighbors in a nearest neighbor

analysis to discern morphological or other similarities between nearby individuals can provide information about the size and connectivity of pockets of cells with distinct morphologies. The spatial identification of vessels and ectopic lymph nodes may have near term clinical implications as key pathological findings and progression projections (Epstein 1993).

Finally, it is likely that landscape pathology, as in landscape ecology, will require mathematical models for simplifying and interrogating complex ecological and evolutionary systems (Johnson 2004). Ecologists and pathologists alike use models to expand testing beyond the time, expense and often feasibility of experimental designs (Jansson 1977; Haslberger 2006). Mathematical models have also had great impact in cancer research in recent years (Rejniak 2010, 2011, Kim 2013). Spatial models in particular have a clear role in interrogating pathological samples (With 2002). One such model not yet translated to medicine deals with ecological niche modeling (Dormann 2012; Araujo 2012). Here niches are defined as the geographic areas necessary for a species to survive. This approach uses matching of individual traits in a species to available resources on the landscape. Furthermore, the niche may limit the distribution of invasive species to a particular region of the landscape (Peterson 2003). These models are often also referred to as bioclimatic envelop models and species distribution models. Here we draw striking parallels to cancer, which grows asymmetrically due to what has been proposed as localized niches of heterogeneous microenvironmental resources (Axelrod 2006, Lloyd 2014). Also interesting is the direct link of the ecological niche model to spatial distribution of resources within regions of a system (MacArthur 1967; Anderson 2002). These models have been highly successful in predicting invasive species spread into new regions of the globe (Rouget, 2003). These concepts may be mapped in landscape pathology images in three dimensions as a topographical representation of an histological image where the third dimension is a measure of multiplexed features of tissues or cells including glucose levels, pH, physical space and oxygen concentrations much like a number of variables including elevation, rainfall, food sources and other considerations might be used in niche models (Figure 3.1 E, F and G).

Summary and Future Directions

In summary, we propose that pathologists have the opportunity to define the Darwinian dynamics within cancers through application of methods and principles of landscape ecology. Using automated image analysis techniques much more precise information can be investigated regarding intratumoral heterogeneity. Furthermore, spatial heterogeneity in these tissue "habitats" can be measured and used to define both prognosis and optimal therapeutic strategies. The latter will require transition from targeted therapies based on genomic analysis of small tumor samples to environmentally- and phenotypically-defined targets based on comprehensive knowledge of the spatial variations throughout the tumor. This is facilitated by automated, high throughput image analysis technologies to identify variations in the physical or molecular metrics of cells and environmental properties.

This approach will require quantitative, reproducible, and comprehensive analysis of cancer as an ecological system. Evaluation of these data as prognostic and predictive biomarkers will require significant effort. However, we propose that this approach to understanding and quantifying intratumoral heterogeneity is necessary to achieve current goals of personalized cancer therapy (Heppner 1983).

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IV. VASCULAR MEASUREMENTS CORRELATE WITH ESTROGEN RECEPTOR STATUS

Abstract

Introduction:

Breast carcinoma can be classified as either Estrogen Receptor (ER) positive or negative by immunohistochemical phenotyping, although ER expression may vary from 1 to 100% of malignant cells within an ER+ tumor. This is similar to genetic variability observed in other tumor types and is generally viewed as a consequence of intratumoral evolution driven by random genetic mutations. Here we view cellular evolution within tumors as a classical Darwinian system in which variations in molecular properties represent predictable adaptations to spatially heterogeneous environmental selection forces. We hypothesize that ER expression is a successful adaptive strategy only if estrogen is present in the microenvironment. Since the dominant source of estrogen is blood flow, we hypothesized that, in general, intratumoral regions with higher blood flow would contain larger numbers of ER+ cells when compared to areas of low blood flow and in turn necrosis.

Methods:

This study used digital pathology whole slide image acquisition and advanced image analysis algorithms. We examined the spatial distribution of ER+ and ER- cells, vascular density, vessel area, and tissue necrosis within histological sections of 24 breast cancer specimens. These data were correlated with the patients ER status and molecular pathology report findings.

Results:

ANOVA analyses revealed a strong correlation between vascular area and ER expression and between high fractional necrosis and absent ER expression (R^2=39%; $p<0.003$ and R^2=46%; $p<0.001$), respectively). ER expression did not correlate with tumor grade or size.

Conclusion:

We conclude that ER expression can be understood as a Darwinian process and linked to variations estrogen deliver by temporal and spatial heterogeneity in blood flow. This correlation suggests strategies to promote intratumoral blood flow or a cyclic introduction of estrogen in the treatment schedule could be explored as a counter-intuitive approach to increase the efficacy of anti-estrogen drugs.

Introduction:

Estrogen (17β-estradiol) is a circulating steroid hormone that binds to intracellular estrogen receptors (ER) after passively diffusing through the plasma membrane (Thomas et al 2011). Estrogen frequently plays a crucial role in breast tumorigenesis by promoting cellular proliferation, and decreasing apoptosis (Fishman et al 1995; Liehr 2000; Devanesan et al 2001; Russo et al 2006; Yager et al 2006). ER expression in breast cancers is used as a prognostic and predictive tool that reliably correlates with the clinical progression of disease and its response to hormonal therapies.

Although the ER status of breast carcinomas is typically expressed as simply positive or negative, there is frequently considerable heterogeneity of ER expression among cells of the same tumor. In fact, typical classification of a tumor as ER positive requires only 1% of the cells expresses ER (Hammond et al 2010). There is now evidence (Viale et al 2007; Endo et al 2011) that the prevalence of ER expression within cells in the same tumor correlates with the degree and duration of response to anti-estrogen therapy.

Our goal here is to investigate the evolutionary and ecological forces that govern heterogeneity of ER expression in breast cancers. Recent studies have demonstrated substantial heterogeneity in cells within the same tumor as a result of intratumoral evolution (Khalique et al 2007; Li et al 2007; Iwasa and Michor 2011; Gerlinger et al 2012; Swanton 2012). Generally, this heterogeneity is viewed as a genetic process in which stochastic mutations generate new populations in an unpredictable if not chaotic process. We note, however, that genetic changes are simply one component of evolution and that intratumoral Darwinian dynamics emerge fundamentally from environmental selection forces that promote phenotypic (not genotypic) adaptations (Alfarouk et al 2013). Furthermore, we acknowledge that a large body of work exists

which addresses the complex dynamics of ER expression in vitro (Pinzone et al 2004 and Stoner et al 2002) and in vivo (Shipitson et al 2007). We embrace these works and do not suggest that phenotypic adaptation alone is sufficient explain variation in ER expression.

Instead, we propose that intratumoral cellular heterogeneity represents a predictable process driven by variations in environmental selection forces leading to predictable and reproducible adaptive strategies. The most obvious source of environmental selection is blood flow which, in most cancers, is spatially and temporally heterogeneous resulting in regions of necrosis in poorly perfused regions.

We propose that ER expression will be observed if it provides an adaptive advantage. Specifically, we propose that ER will be expressed only when estrogen is present in the microenvironment. When estrogen is absent, ER expression represents a needless expenditure of resources and will be selected against. Since the source of estrogen in the breast is typically (although not always) interstitial fluid and moves from the vessels into the cell by a simple reaction diffusion model identical to oxygen, nutrients, etc. (Jiang et al 2002), we propose the hypothesis that ER+ cells will be found in regions of high blood flow while ER- cells will be present in regions of poor blood flow. This results in the prediction that the prevalence of ER+ cells will generally follow the distribution of blood flow. To test this hypothesis we examined regional distribution of ER+ and ER- cells compared to vascular density and regional necrosis within 24 clinical breast cancers of variable ER status and tumor grade.

Material and Methods:

Sample selection and collection

Twenty-four (24) clinically identified breast cancer cases were selected via pathology report reviews by a board certified pathologist (MMB) with the approval of the University of South Florida Institutional Review Board and the Moffitt Cancer Center Scientific Review Committee. Data for each case include the pathologist's estimation of percent ER+ cells, ER stain intensity, and the semi-quantitative Allred score

(Qureshi and Pervez 2010) and histological score. The cases cover a wide spectrum of diagnostic stages including ductal carcinoma in situ (DCIS) (n=11), invasive ductal carcinoma Nottingham Grade I (n=4), Grade II (n=4) and Grade III (n=5). Similarly, the ER status, based on the pathology report, ranged from 0-100% positive and the Allred and histological score were used to create four classification ranges. The Allred score is the sum of a proportion score reflecting the percentage of positive-staining tumor cells (0, none; 1, 1⁄100; 2, 1⁄100 to 1⁄10; 3, 1⁄10 to 1⁄3; 4, 1⁄3 to 2⁄3; and 5, >2⁄3) and an intensity score representing the average intensity of positive tumor cells (0, none; 1, weak, 2, intermediate; and 3, strong). The proportion and intensity scores are added to obtain a total score, which ranges from 0 to 8. (Harvey 1999). The H score is a combination of staining intensity and extent according to the following formula: H score = 1 x % of tumor cells with weak staining + 2 x % of tumor cells with moderate staining + 3 x % of tumor cells with strong staining, resulting in a total score of 0 – 300 (Elston 1998).

The hematoxylin and eosin (H&E) stained sections used for diagnosis from each identified case were retrieved from the department archives and confirmed by the study pathologist (MMB). The blocks identified to have sufficient material and most representative of each case was retrieved from the Cancer Center archives for the purposes of these studies.

Histology

For each of the 24 blocks selected for this study, serial unstained sections were cut at a thickness of 4µm using standard microtomy practices and placed on charged glass slides. The order in which the sections were cut was recorded. The first section was stained with H&E, using standard histological technique. The second serial sections were stained using a mouse monoclonal antibody that reacts to CD34, (#CMA334, Cell Marque, Rocklin, CA) at the stock prediluted concentration. These slides were incubated for 16 minutes at room temperature The Ventana OmniMap anti-mouse secondary antibody was incubated for 12 min. The Ventana ChromoMap kit detection system was used according to the kit protocol and slides were then counterstained with hematoxylin. Slides were covered with #1.5 thick cover glass.

Image Acquisition

Whole slide images (WSI) were produced using an Aperio (Vista, CA, USA) ScanScope XT digital slide scanner with a 20x/ 0.75NA lens. Using the Basler tri-linear array detection, stitching was minimized and the time for most WSIs did not exceed five minutes. Digital WSIs were retained on servers housed within the Moffitt Network Operations Center and accessible on any networked computer via the password protected Spectrum (Aperio) database.

Image Analysis

H&E Segmentation

The commercially available Genie histology pattern recognition platform (Aperio) was trained to classify regions of interest within each of the 13 invasive H&E stained samples. DCIS was analyzed separately to account for the central comedo necrosis common to this non-invasive stage. By manually selecting regions of necrosis, viable tumor and other tissues (including, but not limited to: skin, adipose tissue, and normal margins) the software was trained with the following settings (1000 iterations of uniform distribution with 0.01 as a regularization parameter over 20 stage iterations with eight and three iterations per first and second stage, respectively). Application of this training set over the entire WSI for each patient allowed for computationally derived region segmentation. Each case was carefully quality controlled by a board certified pathologist (MMB).

Vasculature Identification and Quantification

The CD34 stained slides were segmented by the region classification methods described above. Furthermore, the CD34 positive vessels were identified using the Aperio vasculature algorithm with the following settings (Lumen and closed vessels including incomplete vessels with filtering= 2; low= 160; high =210; with stain components .27, .57 and .78 [RGB]). This algorithm was used to export the quantified values for vessel perimeter, area and lumen area.

Statistical Analysis

A partially-hierarchical ANOVA (SYSTAT version 13) analysis was used to test for the effects vessel number, four parameters of vessel size (mean vessel area, mean vessel perimeter, maximum vessel size and mean lumen area) and the percentage of tissue which is necrotic on the tumor grade and ER status of each case. The dependent variables were the different feature data (i.e. vessel number, mean lumen area and percentage of necrotic area) and the independent variables were ER status and tumor grade. The 24 cases being analyzed were the samples.

Results

Vasculature Availability

CD34 positive blood vessels within a manually edited buffer of 300μm from any tumor cell in all directions were identified and individually quantified for each sample. The metrics collected included the number of vessels in the sample. No correlation between vessel number and ER status was elucidated ($R2=7\%$; $p=0.689$).

The vessel size (mean vessel area, mean vessel perimeter, maximum vessel area and mean lumen area)) of the blood vessels was much lower in the samples which did not express ER compared to the ER+ samples as evidenced in Figure 4.1; Figure 4.2 and Table 4.1.

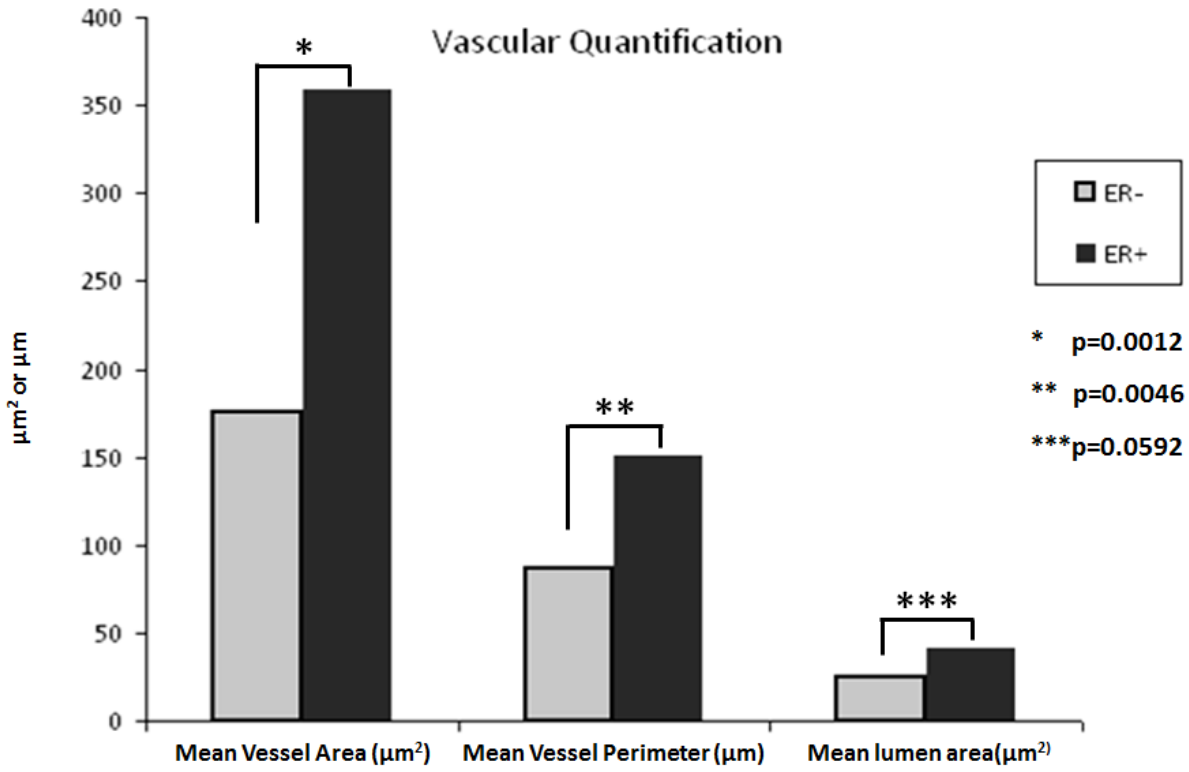

Figure 4.1 Vascular quantification. This graph demonstrates the quantified vascular differences between ER+ and ER- samples. Largely, the ER+ samples exhibit vessel area, perimeter length and lumen size which are statistically significant to demonstrate increased size as compared to the vessels in ER- samples.

	ER-	ER+
Mean Vessel Area (um2)	175.6	358.5
Mean Vessel Perimeter (um)	87.1	151.2
Mean Lumen Area (um2)	24.6	40.7

Table 4.1 Quantified values of mean vascularity for ER+ and ER- patients.

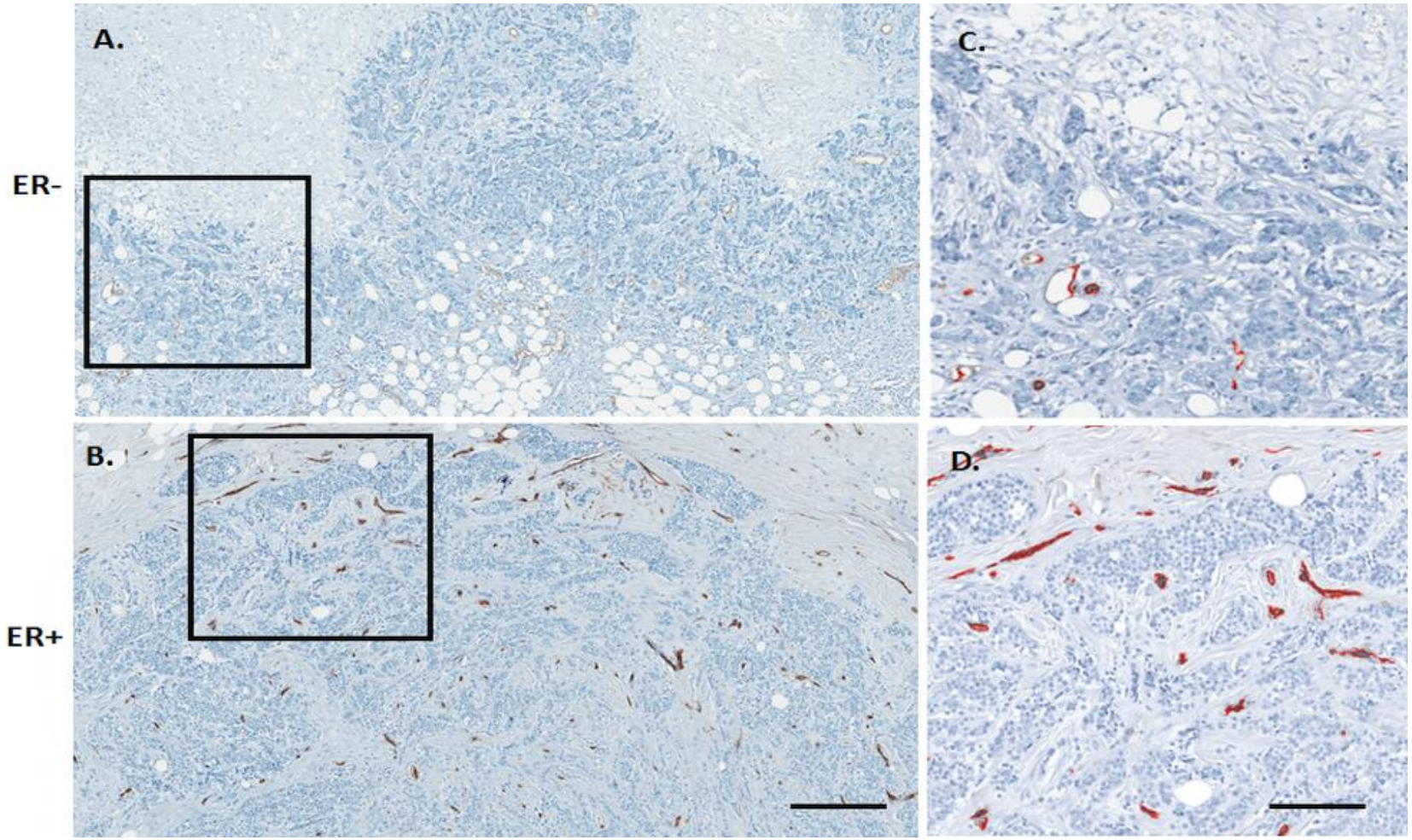

Figure 4.2 Representative vasculature images. A) ER- Grade III invasive breast cancer tumor stains against CD34 with as few as 13 quantified vessels at a region adjacent to the tumor edge. Scale= 800µm. This may be compared with B) ER+ CD34 stained grade III tumor which has as many as 84 vessels in the same area as evidenced by C) and D) which are enlarged views of the inset areas with quantified vessels of each masked in red. Scale bar = 200µm.

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In aggregate the ER- samples exhibited a mean vessel area of 176µm^2 compared to 359µm^2 in ER+ patients (R^2=37%; p=0.003). The perimeter increased from 87µm in ER- to 151µm in ER+ (R^2=40%; p=0.003). Even the maximum vessel area and lumens of the vessels increased from 25µm^2 in ER- to 41µm2 in the ER+ cohort (R^2=18%; p=0.059). To reiterate, the mean vessel diameter of the vasculature of the ER+ regions was about twice that of the vessels if ER- samples in each of the three measures. Vessels identified in the ER- regions never exceeded a mean area over 300µm^2 while 14 of the 18 ER+ cases exhibited a mean exceeding 300 µm^2 with a maximum of 671.4µm^2 (Table 4.2) (R^2=16%; p=0.08). Vessel size was not found to be correlated with disease progression (p=0.295). In other words, vessel size was not statistically different in DCIS samples compared to those of grade I, II or III invasive cancers yet the same metrics of vessel size was highly correlated with ER status. Furthermore, the vascular density (vessels/area) was not correlated with ER status or disease progression (p=0.476).

De-ID Patient #	ER+ or ER-	Percent ER+ cells	Stain Intensity	Allred Score	Histological Score	# of vessels per unit area (um2)	Maximum Vessel Area (um2)	Mean Vessel Area (um2)	Mean Vessel Perimeter (um)	Mean Lumen Area (um2)	Necrotic Area per Tumor Area
Patient 1	-	0	0	0	0	2.12E-04	1301	197.3	91.5	47.5	23.1
Patient 2	-	0	0	0	0	7.97E-05	153	63.1	41.4	7.7	69.3
Patient 3	-	0	0	0	0	3.67E-04	2127	224.4	107.4	21.2	<.5
Patient 4	-	0	0	0	0	5.19E-05	329	102.8	58.2	8.2	16.2
Patient 5	-	0	0	0	0	1.14E-03	12195	287.9	141.5	34.4	22.4
Patient 6	-	0	0	0	0	1.56E-03	8609	178.0	82.9	28.9	14.9
Patient 7	+	90	1+ to 3+	6	150	6.19E-04	13082	482.8	195.7	72.3	33.7
Patient 8	+	95	1+ to 3+	7	155	4.35E-04	4560	307.4	142.1	44.6	1.1
Patient 9	+	95	1+ to 3+	7	165	8.38E-04	12672	282.1	122.8	40.4	<.5
Patient 10	+	90	2+	7	170	7.23E-04	14879	347.7	153.3	34.8	22.1
Patient 11	+	100	1+ to 3+	8	200	4.79E-05	1784	368.6	138.2	33.3	4.7
Patient 12	+	95	1+ to 3+	7	240	5.39E-04	6274	267.6	107.4	61.5	<.5
Patient 13	+	100	2+ to 3+	8	260	5.59E-05	7927	671.4	258.4	16.5	5.2
Patient 14	+	90	3+	8	270	9.18E-04	19704	465.8	191.7	23.2	2.5
Patient 15	+	90	3+	8	270	7.66E-04	16094	341.9	130.9	21.7	<.5
Patient 16	+	100	2+ to 3+	8	270	4.67E-04	1712	178.8	98.7	40.4	1.6
Patient 17	+	100	2+ to 3+	8	270	7.27E-04	19485	436.3	174.1	29.3	<.5
Patient 18	+	100	2+ to 3+	8	280	8.26E-04	4379	221.2	110.9	65.8	<.5
Patient 19	+	100	3+	8	300	2.35E-04	1619	172.4	80.6	30.9	3.9
Patient 20	+	100	3+	8	300	8.78E-05	3673	312.2	152.3	17.6	<.5
Patient 21	+	100	3+	8	300	5.03E-04	8258	271.8	115.1	47.7	<.5
Patient 22	+	100	3+	8	300	4.87E-04	17390	373.7	158.7	52.3	<.5
Patient 23	+	100	3+	8	300	7.74E-04	14857	512.3	234.2	58.8	<.5
Patient 24	+	100	3+	8	300	7.03E-04	11188	438.0	156.7	40.9	<.5
Neg control	NA	NA	NA	NA	NA	1.72E-04	2207	116.1	59.3	6.3	NA
Pos control	NA	NA	NA	NA	NA	9.78E-04	11836	345.8	156.3	22.4	NA

Table 4.2 Summary of all quantification metrics for all patients.

Table 4.2 Summary of all quantification metrics for all patients.

Increasing mean vessel area is associated with increasing tumor perfusion which might represent potential good prognostic value (Kunz et al 2006), and increasing survival rate (Sorensen et al 2012), because tumor oxygenation increases survival rate through improving radiation, chemotherapy and reduces metastatic potentiality (Overgaard et al 1996; Bernards 2003; Clavo et al 2004; Jain et al 2005). These data confirmed that ER-positive tumors have relatively higher average vessel size that might indicate good prognostic value in compare to ER-negative tumors (Teschendorff et al 2007; Fitzgibbons et al 2000).

Necrosis

Necrosis was once often associated with poor vascularization, subsequent hypoxia and the resultant cell death (Vaupel 1977; Bosari et al 1992). Other studies have shown that in fact high vascularization is correlated with necrotic zone expansion (Leek et al 1999). In this study necrosis was segmented from the viable tumor and other tissues including normal margins, adipose tissues et cetera. First, it was necessary to segment the patients by diagnosis so central comedo necrosis commonly found in DCIS patients did not over inflate the results of the invasive population. Regardless of the diagnosis, the viable tumor to necrotic area ratio was calculated for each sample (Table 4.2). Summary statistics were calculated by ER negative and ER positive groups for each diagnostic category. The mean necrosis area in invasive ER- samples was observed to be 24.3%. By contrast, necrosis was quantified to be 4.2% in ER+ tumor samples, demonstrating significantly lower necrosis in ER+ tumors as compared to ER- tumors (R^2=46%; $p<0.001$). Of the ER+ invasive samples 8 of 11 had less than 5% necrosis (Figure 4.3).

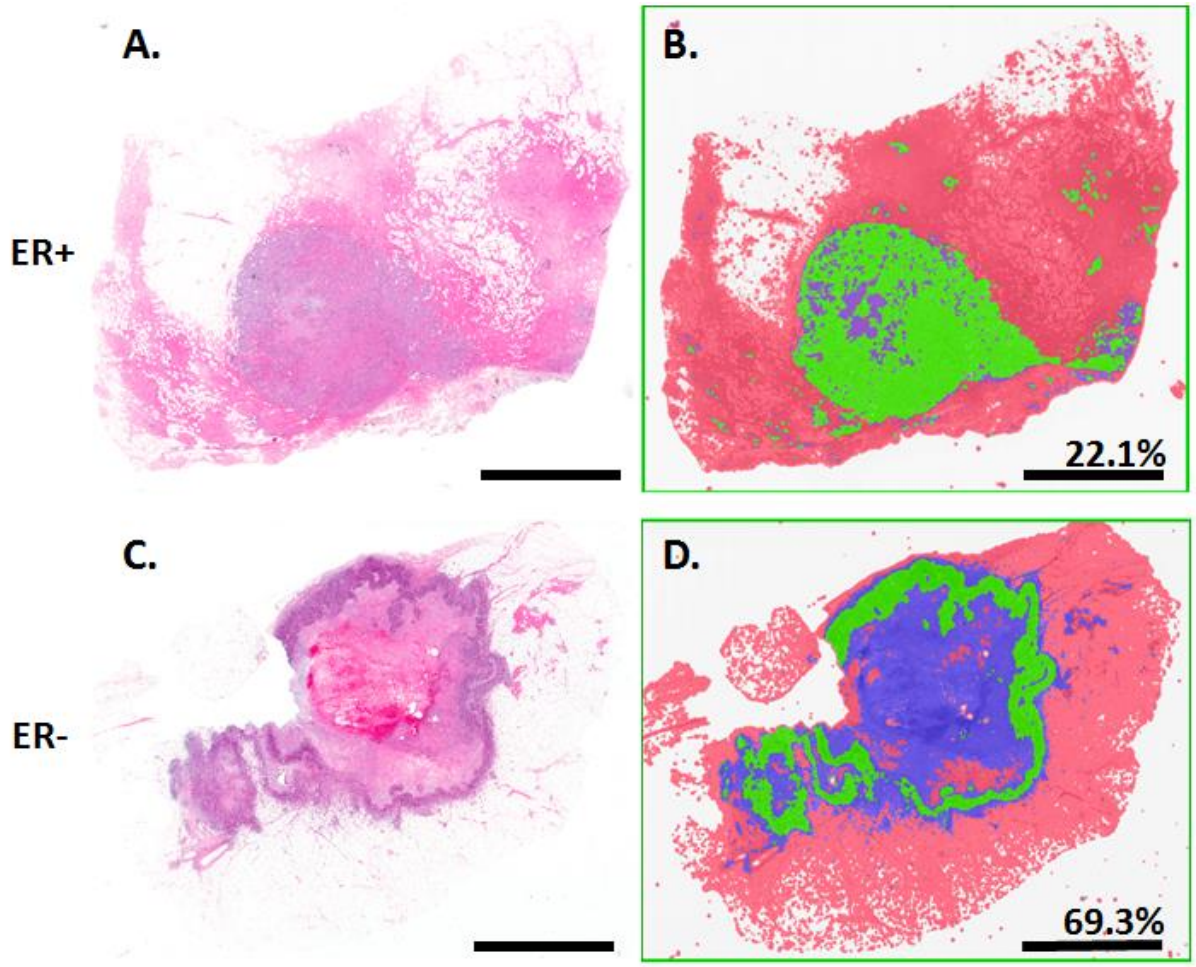

Figure 4.3 Invasive necrosis quantification. Viable tumor is selected via histological pattern recognition software (Genie; Aperio) in green. Other tissues are classified in red and necrotic tissues are classified as blue. In the top panel A) and B) and ER+ sample is presented in comparison to C) and D) in which an ER- sample is shown. Area of necrosis as a percentage of total tumor area (viable tumor and necrotic region) data for each sample is presented. Scale bar = 5mm

Similarly, the DCIS ER- group exhibited 21.3% necrosis area per total tumor area while ER+ DCIS cases were quantified to contain 4.4% necrosis. Of the DCIS ER+ samples 6 of 8 had less than 5% necrosis. These data demonstrate in this sample group that ER-negative tumors have higher necrotic core area relative to viable tumor area. Furthermore, the amount of necrosis in DCIS samples was not found to be dependent on the size of the DCIS or the availability of vasculature outside of the basement membrane of the duct itself. Initially, we hypothesized in DCIS increased necrosis would correlate with ductal size and in turn the distance from the center of the gland to vascular resources. This was not the case in our results.

The average diameter of ER- DCIS with central necrosis was 668μm. The average diameter of ER+ DCIS without central necrosis was 702μm. Examples of the levels of necrosis in ER+ and ER- samples are available in Figure 4.4. Also of interest, in a single case vasculature was observed inside the DCIS. This case was not found to contain necrosis and was ER+ (Figure 4.4E).

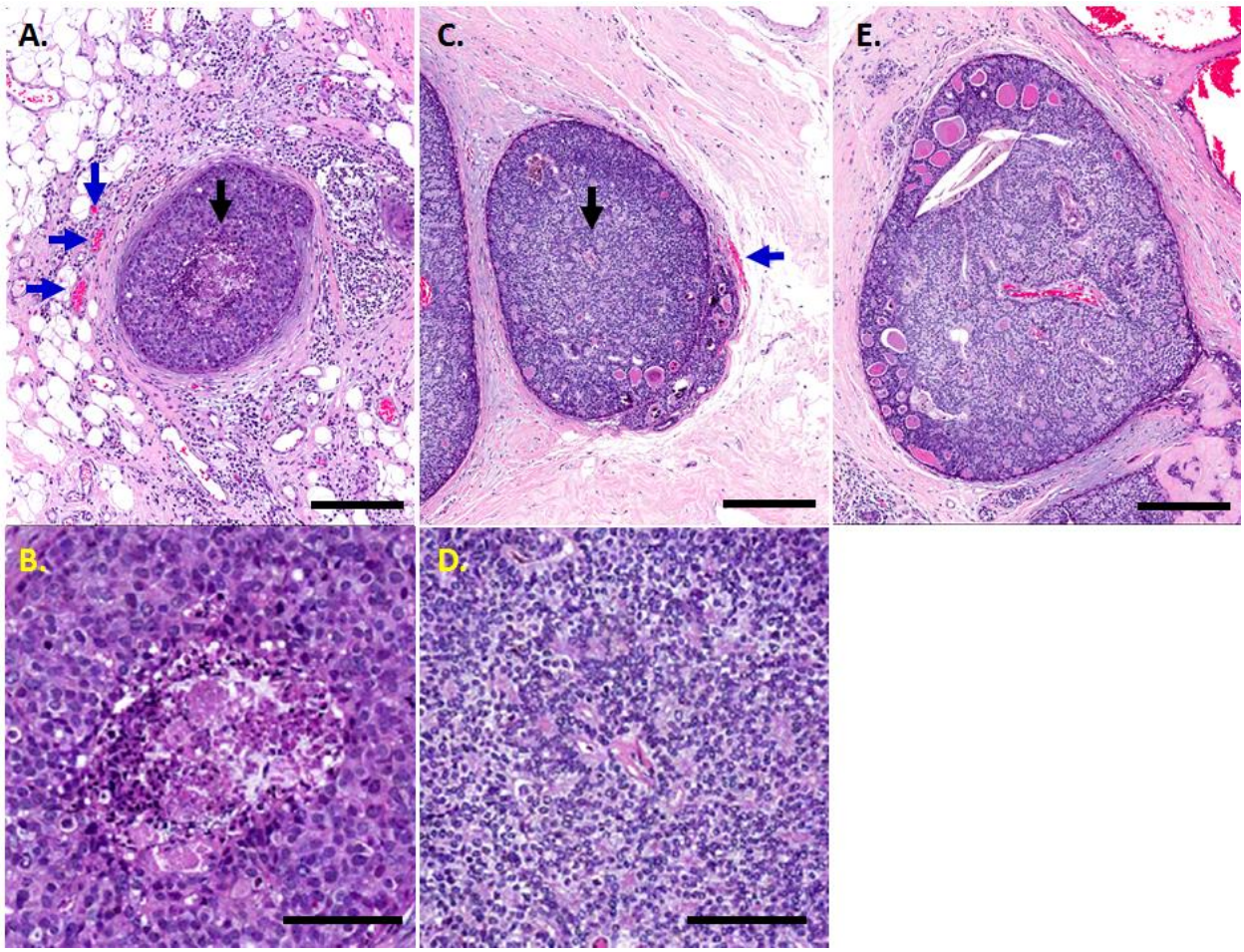

Figure 4.4 Ductal carcinoma in situ localization. This is a DCIS lesion from A) an ER- patient with central necrosis. Note the regionally adjacent vessels outside the lesion (blue arrows) and B) the enlarged image of the central necrosis localized under the black arrow. C) DCIS from an ER+ patient without central necrosis despite the size and distance from the center to vasculature. E) Shows a large (>1mm) DCIS sample with interior vasculature. This sample does not exhibit necrosis and this patient is ER+. Top row scale bars = 250μm; bottom row scale bars = 100μm.

Discussion

ER expression is a useful predictive and prognostic biomarker in breast cancer, however, is often extremely variable even within the same tumor. Similar to cellular heterogeneity found in other cancers (Khalique et al 2007; Li et al 2007; Iwasa and Michor 2011; Gerlinger et al 2012; Swanton 2012), this variation in ER staining is a consequence of intratumoral evolutionary dynamics. Here we address the Darwinian dynamics that might govern cellular ER expression. There are two general components of ER evolution. The first, which cannot be answered with our current study, is the variability of ER expression in the same tumor. Can breast cancer cells adjust ER expression (and other growth factor receptors) so that ER+ and - cells in the same tumor represent a single generalist population that phenotypically adapts to various environments? Alternatively the ER expression could be a relatively fixed property and then ER+ and – cells represent separate, specialist populations. The second component of intratumoral Darwinian dynamics is the environmental factors that are selection forces that define phenotypic fitness. This is the focus of our current work.

Here we propose that spatial heterogeneity in ER expression is the sequela of intratumoral evolution driven not by random mutations but by variations in environmental selection forces and predictable cellular adaptive strategies. We specifically hypothesize that ER expression will correlate with local concentrations of estrogen. Since estrogen diffusion from blood vessels is spatially limited by reaction-diffusion kinetics similar to oxygen and glucose, we predict a correlation between vascularization, necrosis and ER expression (Teschendorff et al 2007; Fitzgibbons et al 2000, Vaupel 1977; Bosari et al 1992). In order to test cell density as a plausible barrier of diffusion we evaluated the mean cell number per mm^2 and did not find any significant difference in the 24 samples evaluated. This suggests cell density alone does not correlate with ER status.

Our results do show that ER+ tumors are associated with larger blood vessels and a lower percentage of tissue necrosis. It should however be noted that differences in CD34 staining may over or underestimate the vascularity due to tumor-specific alterations in the vascular bed such that not all of the endothethial cells may be appreciated. Our initial prediction that vessel number will increase in ER+ samples was not supported. However, the interaction between vessel size and ER status was three times higher than the interactions between tumor grade and ER status and vessel area. Our second hypothesis, that ER status would be inversely correlated with necrosis, was even more strongly supported. This suggests that as ductal carcinoma in situ progresses towards invasion, 1) the larger the vasculature is early in disease progression, the lower the volume of necrosis and 2) if necrosis does not increase with the cancer progression, then ER+ cells are more likely to dominate the population. While the number of patients evaluated in this study is limited, and a larger patient population would be desirable, the number of individual vessels evaluated is on the order of 103 to 104 per patient. For this reason, our results indicate statistical significance to detect differences between ER positive and negative patients.

Furthermore, the amount of necrosis in DCIS samples was not found to be dependent on the size of the DCIS or the availability of vasculature outside of the basement membrane of the duct itself. Initially, we hypothesized in DCIS increased necrosis would correlate with ductal size and in turn the distance from the center of the gland to vascular resources. This was not the case in our results. Also of interest, in a single case vasculature was observed inside the DCIS. This case was not found to contain necrosis and was ER+ (Figure 4E).

Poor vascularization and necrosis was originally hypothesized to be a proxy for hypoxia induced cell death and thus an indicator of low estrogen availability. However, there are a number of plausible explanations (many of which may be responsible in part) why necrotic regions may play a role. In our previous work, we hypothesized that tumor heterogeneity could be predictable similar to that of a riparian zone in a desert environment. Oxygenated phenotypes or relatively highly perfused regions could be equivalent to mesic

species and poorly vascularized (distal from a blood supply) would be equivalent to xeric species (Alfarouk et al 2013). In this regard, ER-positive phenotypes are mesic while ER-negatives are xeric phenotypes.

Xeric habitats are formed by evaporation of water and accumulation of salt which results in salty soil that select for xeric species. In our scenario, hypoxic phenotypes may be shaped by a depletion of nutrients, oxygen and metabolites. That is why ER-negative cells may be adapted to tissue of poor vascularization and higher necrosis. Of course toxification (i.e. salty soil) in such an environment may be another plausible consideration. Regardless, this unavailability of estrogen is one reasonable explanation for estrogen-independent tissue selection. This may be a testable hypothesis in vitro or using techniques including laser capture microdissection to isolate specific regions of high vascularity within patient tumors and evaluating the estrogen concentrations. This is a key future direction for this research.

A significant limitation of our analysis is our inability to measure temporal variations in blood flow. That is, the cyclical and random variations in blood flow which have been extensively observed. These variations will result in temporal variations in estrogen concentration which could alter ER expression. This imprecise link of vascular density and blood flow could result in similar variations in the correlation between vascular density and ER expression.

In conclusion, we find ER expression and metrics of vascular density and blood flow in 24 clinical breast cancers show direct correlations between ER+ tumors and blood vessel size and inverse correlation with necrosis consistent with predictions. This correlation, if confirmed, suggest strategies to promote intratumoral blood flow could be explored as a somewhat counter-intuitive approach to increase the efficacy of anti-estrogen drugs.

Due to natural selection, ER- tumors could be evolving in a way that resists (adapts to) the absence of estrogen. As a future direction, we hypothesize that anti-estrogen therapy (e.g. Tamoxifen) can select for ER-independent cells. In contrast, cyclic introduction of estrogen may improve survival rate by continually altering, rather than unilaterally shifting, toward an ER- population. In other words, this theory suggests that modulation (and not eradication or extinction of certain population) may prove to be an advantageous treatment strategy.

Furthermore, it may be possible that ER+ cells cluster around vasculature and effectively act as a barrier. While this is a future direction of this research and has not yet been tested, it may explain how both populations coexist spatially in a single tumor. More interestingly, it may also be possible that this spatial pattern keeps ER- cells farther from blood vessels where they might enter the bloodstream and form metastatic tumors. ER- may be more prone to metastasize if ER+ cells are less successful invading novel tissues. Future work should determine the spatial relationships around vasculature and the propensity for each population to metastasize. This more in depth assessment of regional distributions of ER+ and ER- cells will be important to understand whether heterogeneous ER staining correlates directly with vessel distribution (i.e. whether ER+ cells congregate nearer to the vessels within a tumor).

In summary, we conclude that ER status is selected for given vascular availability which could have meaningful and exploitable therapeutic decision making implications.

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V. TUMOR CELL EXPRESSION OF HLA CLASS II FACILITATES T-CELL ENGULFMENT

Abstract

Background: Melanoma cells express high levels of HLA class II, cell surface, antigen-presenting proteins, an anomalous phenotype among solid tumors. There has never been a satisfying explanation for how this HLA class II positive phenotype is related to tumor development. Lugini and colleagues demonstrated that melanoma cells have the capacity to engulf T-cells. We considered the possibility that this capacity could be dependent on HLA class II expression. Materials and Methods: We co-cultured melanoma and CD4-positive, labeled, Jurkat-C T-cells. The melanoma cells were transformed with an expression vector for CIITA, the obligate HLA class II gene transactivator. We then assayed for the transfer of label to the melanoma cells. Results: CIITA expression facilitated engulfment of the T-cell material but not material from B-cells. Conclusions: The results suggest a possible mechanism for HLA class II positive melanoma cells in blunting an anti-tumor response and suggest a possible target for melanoma therapy.

Introduction

Melanoma cells exhibit a curious and significant phenotype that is almost unique among solid tumor cells: A large percentage of melanoma cells, represented by either isolated tumor cell lines or different biopsy specimens, are constitutively positive for the HLA class II antigen presenting molecules [1,2,3]. Tumor cells representing most if not all other solid tumors are do not constitutively express HLA class II. Such cells are limited to HLA class II induction by interferon-gamma (IFN-γ), unless the cells have IFN-γ signaling pathway defects, which are indeed common among solid tumor lines [4,5,6,7].

Constitutive expression of HLA class II in melanoma cell lines requires the activation of one of the promoters for class II transactivator (CIITA) by constitutive activation of the mitogen activated protein kinase (MAKP) pathway [8,9]. MAKP is considered an important driver of tumorigenesis in melanoma. CIITA expression may occur due to the direct effects of malignant transformation. However, it has also been proposed that constitutive CIITA expression in melanoma cells is due to fusion of tumor cells with CIITA-constitutive macrophage [10,11,12]. CIITA expression has been linked to a reduction in apoptosis [13]. In fact, other

solid tumors besides melanoma express CIITA, but without the corresponding high level of HLA class II seen in melanoma cell lines and in many patients biopsies [14]. CIITA is involved in B-lymphoma translocations, but with the apparent effect of HLA class II down-regulation , proposed as a possible mechanism of blunting the anti-tumor response [15].

Materials and Methods:

Cells and HLA-DR staining: Cell culture and antibody staining for flow cytometry for detection of HLA-DR were as described [16]. Briefly, cells were maintained in RPMI, 10% fetal calf serum, penicillin, streptomycin and glutamine. To assay for surface HLA-DR, cells were scraped, recovered by centrifugation, and resuspended in 1% human serum in PBS to block Fc-receptors. Commercially available PE-labeled, anti-HLA-DR or isotype control were added according to vendor's instructions for one hour at 4o C, cells were recovered by centrifugation and resupended in 1% human serum in PBS for flow cytometry.

Jurkat-C eFluor-670 labeling and cell co-culturing for fusion assays: Jurkat-C were labeled according to vendor's (eBioscience) instructions then immediately added to a 50% confluent plate of adherent cells. Approximately 1 million labeled Jurkat-C cells were added to 10 ml of media in a 100 mm plate of adherent cells. Cells were co-cultured 48 hours before assay for adherent cell uptake of eFluor-670, either by microscopy or flow cytometry.

Microscopy: Photomicrographs were acquired with a Zeiss (Zeiss Microsystems Manheim, Germany) AxioObserver inverted fluorescent microscope; enclosed in a full incubation environment controlled at 37°C and 5% CO2; with a Zeiss MrM monochrome CCD detector; through a 20X/0.5NA Plan NeoFluor dry objective; and viewed, prepared and exported with the Zeiss Axiovert v4.8.3 software suite. Phase contrast and Cy5 (620nm/60 excitation; 700nm/75 emission) images were acquired sequentially and the fluorescent images were pseudo-colored red. Exposure time (1500ms), gain, offset, and similar acquisition settings were identical for all samples. Each image set was repeated five times.

Image Analysis: Raw Zeiss .zvi files were exported as merged and individual channel uncompressed .tif files. The merged phase contrast and red pseudo-colored images were imported into Definiens Developer v2.0 (Definiens, Munich, Germany). A morphological filter rule set was designed to isolate and classify the small, round, suspended T-cells and the larger, elongated, flat cancer cells. The cancer cells were further segmented into individual cells using the phase contrast membrane definition as a border to a shrink and grow process. The number of adherent cancer cells was quantified for each sample for all five repetitive image sets. The amount of red positivity for each cell was subsequently quantified continuously and all cells with a region larger than 10 pixels and also presented with expression of red fluorescence greater than a threshold of 150 (dynamic range for the 8 bit image) were considered red positive. The percentage of red positive adherent cells to all adherent cells was calculated.

Flow Cytometry: Samples were assayed with a BD LSR II flow cytometer. Tumor cells and lymphocytes were distinguished on Forward and Side Scatter plots. eFluor-670 labeled cells were excited with a 633nm laser and the fluorescence emission was detected in the 660/20 nm range. Results were analyzed with BD Facsdiva Software V6.1.3, mean and median fluorescent values associated with cell populations were exported to Microsoft Excel files.

Results

Lugini et al [17] observed that melanoma cells have the capacity to consume T-cells, however, their work did not suggest a mechanism. This function adds a new dimension to the ways tumor cells interact with the immune system, in addition to evasion or co-option [18], and provides a role for HLA class II that could be opposite HLA class II's presumed role of improving the anti-tumor response in other settings [15,19]. Here, we investigate the possibility that the common expression of HLA class II on melanoma cells provides the trait permitting T-cell engulfment by the melanoma cells. This hypothesis is difficult if not impossible to test using HLA class II blocking antibodies, because of the capacity of such antibodies to facilitate HLA-DR mediated apoptosis [16,20]. Thus, we transformed (originally HLA class II-negative) melanoma cells using

an expression vector for CIITA and verified expression of HLA-DR, the canonical HLA class II protein (Figure 5.1) [16].

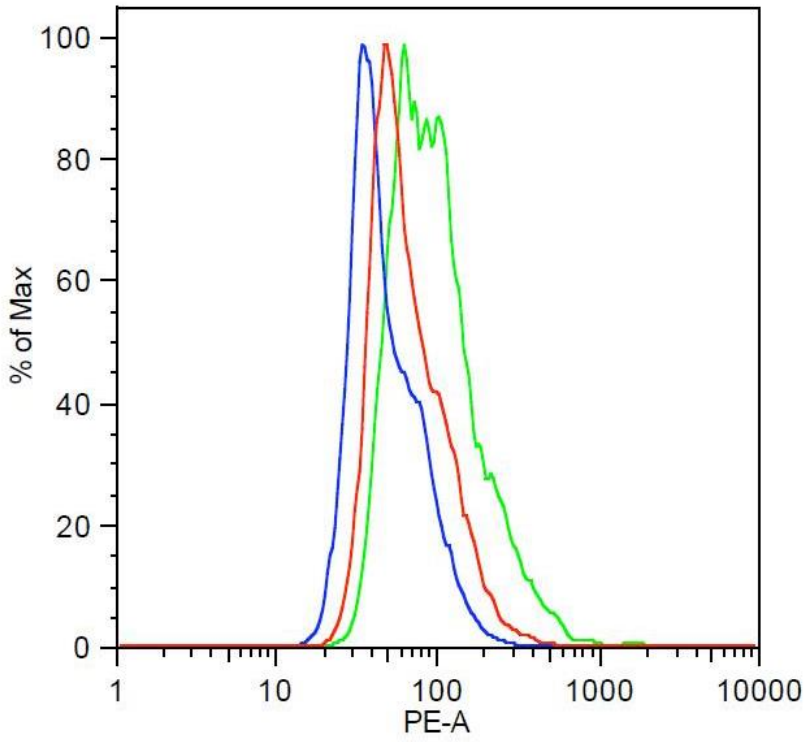

Figure 5.1 Detection of HLA-DR (PE) in CIITA transformants by flow cytometry. Cells were stained with PE-labeled, commercially anti-HLA-DR exactly as described [23,24]. 66-CIITA (CIITA transformant of H2009 NSCLC; right histogram, green), CIITA-3 (middle histogram, red), and RSV2 (left histogram, blue).

We labeled CD4-positive, Jurkat-C cells with eFluor-670 and co-cultured these cells with CIITA-3, transformant of 1286 melanoma cells, isolated as described in ref. [16]. Previous work has indicated that CIITA-3 not only expresses HLA-DR (Figure 5.1), but the HLA-DR is functional in an HLA-DR dependent apoptosis assay [16]. We also mixed the labeled Jurkat-C cells with H2009 non-small cell lung cells (NSCLC) that had been transformed with the CIITA expression vector and thereby express HLA-DR (Figure 5.1), as well as with the control, 1286 melanoma cells, transformed with an empty expression vector, termed RSV-2. We observed uptake of the eFluor dye by the adherent cancer cells, as indicated in Figure 5.2; and we quantified the microscope images, which indicated that both CIITA transformants had more eFluor dye than the RSV-2 cells (Table 5.1).

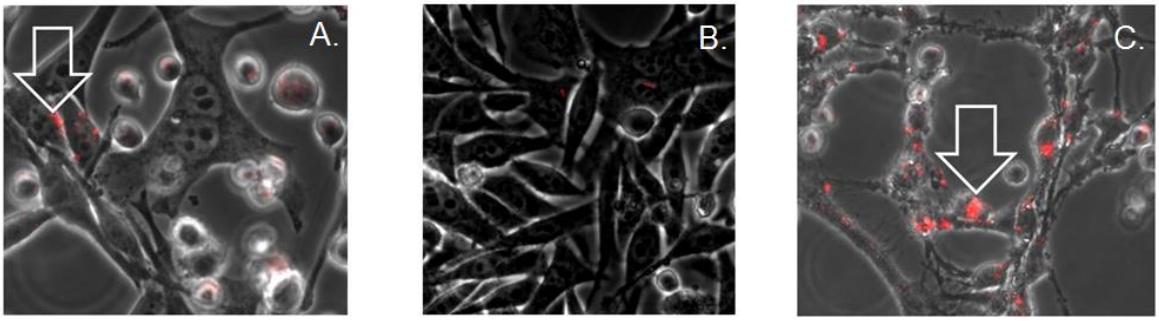

Figure 5.2 Microscopic detection of eFluor-670 in adherent tumor cells at 200x total magnification in live cells. Adherent CTIIA-3 transformed cells display significant internalized punctate red fluorophore (white arrow) (A); whereas RSV-2 (empty vector, control transformed) cells confirmed a lack of fluorophore detection (B). 66-CIITA similarly presented with internalized red punctate staining (C).

Table 5.1 Percent of eFluor-670 labeled tumor cells following incubation with eFluor-670 labeled Jurkat-C T-cells.

Cell line	Percent labeled	*p*-value compared to HLA class II negative cells
CIITA-3 (CIITA transformant)	9.1	.003
RSV-2 (empty vector transformant)	4.2	NA
66-CIITA (CIITA transformant of H2009 NSCLC)	45.8	1.25 E-07

Table 5.1 Percentage of eFluor-670 labeled tumor cells following incubation with eFluor-670 labeled Jurkat-C T-cells

The CIITA-3 melanoma cells displayed fluorescence in 9.1% of the cells compared to 4.2% of the RSV-2 HLA class II negative cells ($p<0.01$). Remarkably, 45.8% of H2009 CIITA transformed cells expressed eFLuor dye, consistent with the higher level of HLA-DR expression in these cells (Figure 5.1) ($p<0.0005$ compared to 4.2% RSV-2). However, the H2009 cells are not melanoma cells, and despite the increased level of HLA-DR on these cells, it is possible other factors play a role in the dramatically high level of eFluor uptate, such as a higher level of H2009 phagocytosis.

We next assayed for eFluor uptake by flow cytometry (Figure 5.3), which indicated that the presence of HLA class II led to a 30% increase in uptake of Jurkat-C eFluor by the CIITA-3 melanoma cells but had no effect on uptake of material from two eFluor labeled B-cell lines, Raji and Mann, which lack CD4 or other HLA-class II binding, T-cell receptor components.

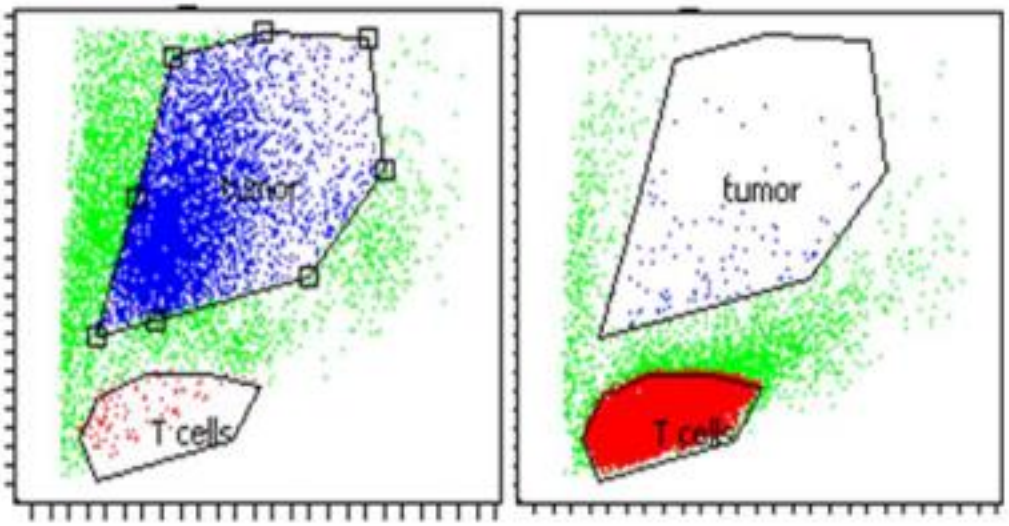

Figure 5.3 Detection of eFluor-670 uptake by tumors using flow cytometry. Flow cytometry gates indicating detection of eFluor-670 in the adherent tumor cells versus Jurkat-C T-cells. The two plots (3A) represent isolated cells indicating the function of the gates for detection of each cell type, respectively, and in particular, indicating lack of gate overlap. Bar graphs (3B) indicating increased level of eFluor-670 uptake by the CIITA-3 cells, compared with the RSV-2 cells, in two separate experiments (where the value for the RSV-2 cells is normalized to one, indicated by arrow head, y-axis).

Discussion

The above data are consistent with the conclusion that HLA class II expression facilitates uptake of Jurkat-C T-cell material, raising the question of whether HLA class II expression on melanoma cells in patients would affect the T-cell response against the melanoma cells? Also, these data provide a potential mechanism for theories of metastasis, whereby lymphocyte-tumor cell fusions are at the heart of tumor cell migration [10,21,22]. However, it should be noted that CIITA expression can lead to other melanoma cell alterations, and despite the work by Lugini and colleagues [17], there are potential, alternative processes that could lead to the transfer of eFluor dye to the melanoma cells, such as exosome mediated transfer or other processes of debris engulfment.

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VI. IT'S ALL THE RAGE: AN EVOLUTIONARY ECOLOGY PERSPECTIVE ON MEXTLGLYOXAL AND ITS CONSEQUENCES FOR LOCAL AND SYSTEMIC TOXICITY IN CANCER PATIENTS

Introduction

An increase in epigenetic studies of cancer has revived Lamarkian theories of evolution [Handel 2010]. While Lamarck's theory of the inheritance of acquired traits may not accurately explain mechanisms of inheritance in organisms, new insights can be gained when tumor cells are seen as evolutionarily and ecologically dynamic units of natural selection capable of adapting to their environment. A model that maps the role of methylglyoxal (MGO) in cancer development is proposed to explain tumors' production and use of toxic metabolites and the impact of these metabolites on tumor evolution, the integrity of the tissue environment, and, ultimately, the patient. The main purpose of this analysis is to review a model by which tumor cells may inadvertently poison their host via amplified metabolic pathways.

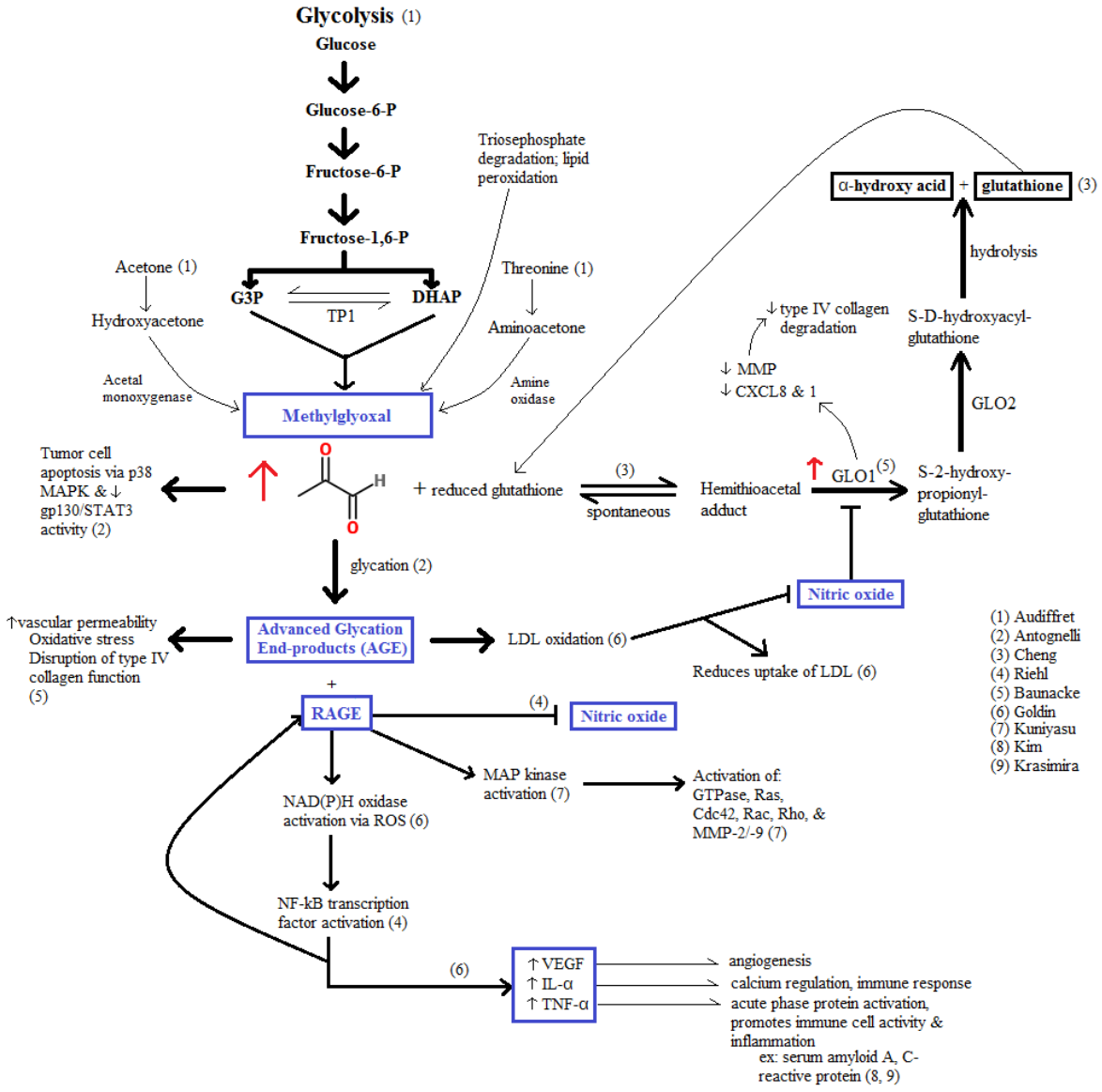

Figure 6.1 MGO Model with emphasis on the key chemical, enzymatic and receptor agents which drive the model from MGP, a byproduct of glycolysis, to a cascade of actions which influence the cellular environment and cellular fitness.

A classic example of Lamarck's argument that organisms respond to changes in the environment by altering the degree to which a feature is used is the direct selection for anaerobic respiration which initiates a feedback process between tumor cells and the surrounding environment, thereby promoting tumor development. End-stage cancer, then, may be the culmination of compounding effects of tumor cell

adaptation to changes in the environment induced by unintended consequences of toxic metabolites and by the exploitation of associated metabolic pathways to increase survival and proliferation.

Methylglyoxal (MGO) is a by-product of multiple metabolic pathways- most notably glycolysis. MGO is a reactive aldehyde which causes severe cytotoxicity, In the MGO model described, early adaptation to tolerate MGO toxicity are observed. Tumors adapt to increased MGO production by amplifying glyoxalase-1 (GLO1) but also exploit MGO for the production of advanced glycation end-products (AGE) and their receptor, RAGE. AGE and RAGE promote increased VEGF, IL, TNF, and MAP-K activity, as well as reduction of LDL uptake and NO regulation. An evolutionary ecology framework may draw attention to these altered metabolic pathways, such as the proposed MGO model. This pathway and associated toxins may have a greater impact on a patient's well-being than the physical tumors themselves.

Background

Views regarding cancers have changed over the decades, and continue to do so, as new technologies and findings help uncover some of the mysteries of cancer development and progression. A commonly cited theory, as proposed by Douglas Hanahan and Robert Weinberg, posits that normal cells that transform into tumor cells do so by undergoing six changes considered the 'Hallmarks of Cancer': (a) cells proliferate without external control; (b) there is reduced response to inhibitory factors; (c) dysfunction of components contributing to apoptosis occurs; (d) angiogenesis is promoted; (e) cell life is prolonged; and (f) cells eventually invade other tissue and metastasize [Hanahan 2000; Hanahan 2011]. Although the standard theory characterizes many aspects of observed tumor cell behavior, it underestimates the influence of the microenvironment and the interactions tumor cells have with surrounding normal cells, with each other, and with immune cells. It also fails to recognize that a tumor consists of a population of evolutionarily dynamic and genetically distinct cells [Michor 2005; Merlo 2006; Crespi 2005].

From this perspective, tumor cells follow Darwinian dynamics of trait evolution and population dynamics [Gatenby 2003]. Tumor cells become the units of natural selection; they do not exhibit multi-cellular

behavior because they proliferate rapidly and select for factors that increase their fitness, not the whole organism. Tumor cells may be considered units of natural selection because they have the following attributes: a) populations of cells have phenotypic variability; b) populations are subject to the effects of the microenvironment; c) cells that acquire certain traits are capable of passing those traits to daughter cells by mitosis; and d) members of the population have different levels of fitness and that fitness is related to phenotypic traits [Greaves 2012]. Mathematicians have used these concepts to construct theoretical models describing the evolution of a population of tumor cells by taking into account several contributing variables such as the rates of tumor cell birth and cell death, nutrient diffusion, the effects of inhibitory factors, destructive interactions with other cells, etc. [Bellomo 2000].

As the mathematical models suggest, the tumor as a population of cells is influenced by changes that occur in the environment. Populations that follow Darwin's principles of natural selection are subject to competition for limited space (tumor cells surrounded by highly structured, normal tissue) and limited resources (low glucose and/or oxygen levels, for instance) [Lorusso 2008]. Survival against competition from other tumor cells and normal cells is one factor that may drive tissue invasion. This can be accomplished through angiogenesis, or by the formation of gaps in the habitat [Daoust 2012]. In fact, tumor volume is typically limited to only a few mm3 in the absence of adequate vascularization – angiogenesis enables tumors to establish a reservoir where access to nutrients, pathways to new tissue sites, and immune cells becomes possible [Lorusso 2008].

The ability to vascularize and interact with normal tissue cells that eventually compromise the integrity of the normal infrastructure may depend on the stimulation and promotion of local inflammation and other environmental perturbations. The maintenance of inflammation at the tumor site is critical to further development of a tumor [Beutler 1988]; consequent gaps in the microhabitat from necrosis of nearby normal tissue due to the effects of tumor metabolism can promote metastasis. In fact, metastasis is positively associated with large gaps that are close to each other [Daoust 2012]. The tumor microenvironment consists of endothelial cells, fibroblasts, smooth muscle cells, macrophages, T cells, neutrophils,

eosinophils, and other cells associated with the immune system [Condeelis 2006; Spaeth 2008]. Immune cells have been found to play a substantial role in the initiation of angiogenesis. Factors involved in the promotion of angiogenesis include interleukins (IL), endothelial growth factors (VEGF), and matrix metalloproteinases (MM-2, -9) [Carmeliet 2000]. An inflammatory response to a tumor is not short-lived – rather, heightened immune system activity is prolonged as tumor cells recruit leukocytes and macrophages. Chronic inflammation then leads to the accumulation of cyclooxygenase reaction products and increased nitric oxide production, which have been found to promote proliferation [Grimm 2013; Vakkila 2004].

A protein believed to be crucial in initiating inflammation is high-mobility group box 1 (HMGB-1), which is normally responsible for regulating access to DNA transcription. Releasing HMGB-1 from necrotic cells – but not cells undergoing apoptosis – stimulates immune cell recruitment. It has also been found that expression of the receptor for HMGB-1, which is also known as the receptor for advanced glycation end-products (RAGE), is markedly increased in several types of cancers including hepatomas, mesothelioma, prostate cancer, breast cancer, colorectal carcinoma, and, in one study, in all patients with advanced metastatic cancer [Ishiguro 2005; Kuniyasu 2002]. RAGE is typically expressed by neurons, endothelial and vascular smooth muscle cells, immune cells, and by osteoblasts; it helps regulate cellular responses to stress and damage by activating pathways promoting inflammation. But, it also contributes to endothelial cell dysfunction and is often overexpressed in other diseases such as diabetes, arthritis, atherioscIerosis, and Alzheimer's disease [Riehl 2009; Wautier 2004] for reasons believed to be related to the release of stress of elevated sugar phosphate concentrations [Giacco 2010].

Lamarck proposed that use or dis-use of traits in response to changes in the environment could become heritable and passed along to offspring. Although such a pathway towards evolving adaptations may not apply to multi-cellular organisms, the theory may be applied to tumor cells because they undergo changes to their genome other than loss of function/gain of function mutations such as amplifications and/or inhibition of genes. Due to tumor cells' high energy demands, there is a direct selection for glycolysis and, as a

result, the rate of glycolysis increases relative to aerobic respiration [Seyfried 2013; Ward 2012]. This is termed the Warburg effect, and it appears to become heritable, perhaps via epigenetics.

With an increase in glycolysis production, however, there is a tradeoff – the increase in production of methylglyoxal (MGO), a metabolite that is toxic to the cells that produce it [Antognelli 2013; Bair 2010; Thornalley 2003]. The MGO model offers a link between early tumor development and metastasis via Lamarck's theory and the habitat gap formation hypothesis. Although initially methylglyoxal production may kill some tumor cells, tumor cells – unlike normal cells – are capable of adapting to that change. Tumor cells appear to evolve tolerance and even the ability to re-purpose MGO for other fitness enhancing metabolic pathways. Methylglyoxal reacts non-enzymatically to form advanced glycation end-products (AGE) under typical conditions [Lv 2011]. This model suggests that the described metabolic changes lead to unintended toxic consequences, which in highly glycolytic tumor cells leads to a fitness advantage over normal cells. Tumor cells may adapt to manage the toxicity of MGO which consequently changes their environment. Later, tumor cells may re-purpose this pathway to promote immune cell recruitment. This recruitment maintains inflammation and promotes vascularization. As an added benefit to the tumor cells, the release of AGE and RAGE into the interstitial fluid overwhelms and poisons normal cells that do not possess the tumor cells' trait conferring tolerance. We hypothesize that this cytotoxic cascade promotes some of the symptoms and discomforts that patients experience are such symptoms are not due solely to the tumor mass itself (unless in the case of obstruction). Failing patient health may be due to the compounding effects of toxic metabolites released or produced when tumor cells evolve to utilize new metabolic pathways, evolve tolerance to cytotoxins and evolve to exploit existing compounds in new ways.

Model Formulation

Tumor cells have greater metabolic demands than normal cells – glycolysis provides tumor cells the opportunity to keep up with cellular demands. Reduced mitochondrial function and/or dysfunction in tumor cells and increased reliance on glycolysis are adaptations that benefit tumor cell metabolism [Gatenby 2004]. An unintended consequence of enhanced glycolysis is the increase in production of methylglyoxal,

toxic to both the cells producing it and to surrounding normal cells. Some tumor cells (and normal cells) may die in a non-apoptotic manner because of the increasingly acidic/toxic and hypoxic environment and because of the increased MGO. Those tumor cells that adapt to this environment and amplify the glyoxalase 1 (GLO-1) gene are able to survive and proliferate, in a manner akin to Lamarckian evolution. Epigenetic changes acquired by the tumor cell to tolerate the degraded environment are passed along the daughter cells, even to the point where such changes become genetically fixed. Those cells that die do not package or degrade toxic metabolites for disposal. Instead, the tumor cells cease apoptosis and die in a "necrotic" manner spilling a variety of metabolites and biochemical into the tumor environment. HMGB-1 released from necrotic cells and surrounding tissue cells may stimulate the immune system, initiating an inflammatory response (Figure 6.2).

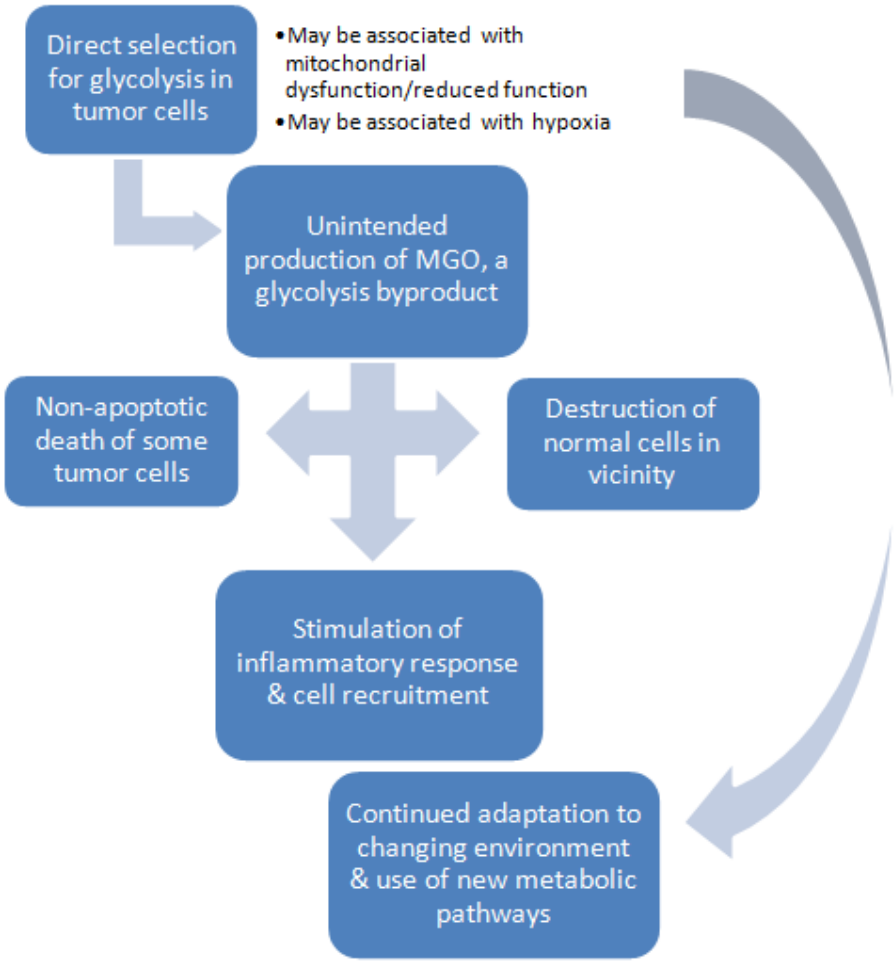

Figure 6.2 Suggested pathway from glycolysis to adaption to the novel environment.

Generally, MGO is neutralized as part of the whole organism's homeostatic process. But in tumors, not all of the MGO may be neutralized by GLO-1/-2. Firstly, the glyoxalase system is expensive to the cells that use it: its subunits require zinc, and the enzyme requires electron carriers and glutathione to carry out its function. Furthermore, tumor cells produce MGO faster than available GLO-1/-2 can neutralize it [Thornalley 2003]. The production of methylglyoxal is not limited to glycolysis. Lipid peroxidation, the degradation of triosephosphates, and enzymatic reactions involving acetone and threonine also contribute to the production of MGO (Figure 6.1) [Audiffret 2008]. The metabolite facilitates the production of AGE via glycation reactions to proteins and lipids in and outside of the cell in the extracellular matrix.

AGE cross-linked to the extracellular matrix is virtually non-degradable. Because there is no way of naturally disposing of AGE, the molecules accumulate. An intended result of increased AGE production is the over-expression of RAGE. In tumors, which are highly glycolytic and produce and accumulate AGE, RAGE activates VEGF, IL-α, TNF-α, acute phase proteins. Together, RAGE and AGE activity promote formation of blood vessels in the tumor, increase the rate of proliferation, destroy the vital structure of surrounding tissue and influence the immune response. These actions increase gap formation in the tumor, creating habitat for further tumor cell proliferation. Via the RAGE and AGE pathways, tumor cells literally create space, amplify resource availability, and reduce competition from normal cells or tumor cells that fail to evolve tolerance to these toxins. The advantages to tumor cells involved in the re-purposing of AGE and RAGE may create a feedback of ever greater production and tolerance. The net effect will be increased cytotoxity to the surrounding normal tissue, system-wide hemotoxicity, and reduced kidney function. The expansion of ill-effects of the AGE and RAGE metabolic pathways beyond the tumor may promote many of the symptoms of pain, loss of appetite, and lethargy associated with cancer. The tumor itself may not cause these symptoms directly in the absence of spreading metabolites.

Discussion

Cancer symptoms may not necessarily be due to the tumor mass itself, except in cases in which obstruction occurs. Instead, as tumors develop over time following Darwinian principles of evolution, they adapt to survive the detrimental effects of – and possibly utilize, as in the case of methylglyoxal – toxic metabolites.

In some cancers such as gastric, breast and prostate cancer, mesothelioma, renal cell carcinoma, and ovarian and lung cancers, these may be responsible for the deterioration of organ function and eventual systemic failure in patients. Given the range of diseases AGE and RAGE are associated with, research on their role (as well as methylglyoxal's role) in cancer development can be beneficial to understanding the interactions tumor cells have with immune cells, normal cells of surrounding tissue, components of the vascular system, and other tumor cells. Blood work or analysis of cell interstitial fluid can provide information about the kinds of toxic metabolites or levels of associated compounds present in patient samples. In addition, nitric oxide regulation, inhibition of RAGE, a mechanism that can interfere with AGE binding, genistein-like compounds that can trap methylglyoxal efficiently and bicarbonate therapy are just some of many potential methods that may be developed or used to help improve patients' well-being.

Cancer may not be a curable disease, but in understanding the roles of toxins and toxic metabolites in the progression of cancer and developing counterstrategies to them, treatment can be directed toward managing symptoms and regulating levels of metabolites crucial in promoting tumor cell proliferation. Managing the toxicity caused by metabolites may be as important as managing the tumor itself. In fact, focusing on the tumor's destruction itself may inadvertently add to the toxins released into the blood and spread to other parts of the body. It has been anecdotally observed that often the tumor itself does not induce patient death by virtue of organ system failure. Rather, a system-wide collapse of function and homeostasis may cause the pain, lethargy, loss of appetite and other symptoms that may precede death. Cause of death may be the emergent consequence of billions of cancer cells within the tumor releasing toxic metabolites for very selfish and limited purposes.

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VII. A MULTIDISCIPLNARY REVIEW OF THE ECOLOGICAL FRAMEWORK OF METASTATIC CANCER

Abstract

A neoplasm is an ecosystem of biotic and abiotic factors. The biotic component consists largely of tumor cells and angiogenic blood vessels as well as normal tissue cells and inflammatory responses. The abiotic component consists of oxygen, glucose and other important chemical and physical aspects of the environment that effect the living cells. Within the biotic mix of any given neoplasm are clusters of heterogeneous tumor and normal cells that compete for abiotic resources such as space and nutrients. Given the high competition and limited resources, it is reasonable to expect tumor cells to use a strategy of dispersal and colonization of secondary sites. In oncology this mechanism is termed metastasis and it is one of the most clinically significant prognostic indicators for long-term patient survival.

Similar concepts have been studied for centuries in the natural world. This material is meant to apply principles of invasion ecology to speculate and hypothesize about the characteristics and types of tumor cells that one might look for in primary tumors that are likely to go on and produce metastases. This may elucidate principles of metastasis which are not fully understood and aid in the introduction of new ideas for clinical treatment. In this literature-based review, concepts of invasion ecology, are used to frame metastasis within existing theories of ecological invasion. There may be several different strategies which heterogenic tumor cells may employ to successfully populate distant body sites. We propose five major types of tumor cell strategies that may cause the metastatic cascade. We term these “ecological engineer”, “ecological pioneer”, “penguin”, “treasure-seeker” and “canoeist”.

Introduction

Metastatic cancer is an invasive process in which primary cancer cells travel long distances through the body and compete with the body’s native cells for survival [Poste 1980]. Consistently, sites of metastatic cancer share a well-defined and finite number of patterns of invasion. Here, a general outline is presented for the four well-established stages for metastasis based on the invasion process following shedding tumor

cells from primary tumor, 1) intravasation (invasion into the blood stream), 2) circulation and evasion of immune system, 3) extravasation (withdrawal from the blood stream), and 4) establishment and angiogenesis [Paterlini-Brechot 2007].

An invasive species in ecology is an organism/species that is non-native to the environment, and capable of surviving and flourishing in that environment. If introduced, it is able to successfully colonize and expand into other sites sometimes far away from its initial establishment site [Lockwood 2007]. Not all invasion events are successful, much like the invasion events of a circulating tumor cell. It is the failures along the path of invasion that may prove useful in understanding metastatic cancer and how to slow or prevent the dissemination of tumor cells to new organs. To make invasions fail, we aim for a deeper understanding of the full process of invasion as well as the qualities or characteristics that the invasive species may have that enable them to succeed. Akin to metastasis, the stages of ecological invasion are generally: 1) transport, 2) colonization, 3) establishment, and 4) landscape spread [Theoharides 2007]. These stages are analogous to the steps in the metastatic cascade described above [Gatenby 2009].

The goal of this review is to provide a framework for addressing the following question - can the principles and concepts from invasion ecology meaningfully contribute to a novel framework for understanding the metastatic cancer process and introduce new ideas for clinical treatment? Can we characterize the properties of tumor cells that may impede or facilitate migration and establishment in distant organs? And, are there discernable patterns and associations between the organs of the primary tumor (donor community) and the organs subject to metastasis (recipient community)?

Invasion ecology is a fast expanding ecological discipline, with the number of articles on the subject having grown exponentially since the early 1900s [Lockwood 2007, 2009]. Using ideas and principles from invasion ecology as a framework for understanding the metastatic cancer process may help elucidate areas that are not well understood and introduce new ideas for therapy design. There are parallels today between

cancer and the study of evolutionary/invasion ecology. Several decades of research have broadly supported Nowell's description of cancer as an evolutionary system [Nowell 1976; Merlo 2006]. Since this important work and others, several researchers have studied cancer as an evolutionary and ecological process.

This review will concentrate on invasion ecology as the framework for the metastatic processes of cancer. We will first provide an introduction to invasion ecology and discuss cancer through an evolutionary and ecological approach. Invasion ecology can be used, at a minimum, to investigate metastasis by applying several currently debated theories for ecological invasion. The second portion of this review will outline invasion ecology concepts including enemy release, propagule pressure, tens rule and biotic resistance hypotheses [Keane 2002; Colautti 2006; Williamson 1996]. Third, it is important to note that the success of invasive species, such as tumor cells, depends heavily upon a series of filters. The important filters that accompany the stages of metastases that will be reviewed include dispersal-limitation, recipient-limitation, organ-specific signaling, number of circulatory rounds before arrest, and nutrient-limitation [Fidler 2003]. An explanation as to which filters may select for which types of tumor cells, and where in the body we might find such tumor cells will be discussed. Finally, in the fourth section, we highlight the importance of tumor cell heterogeneity. We propose five different metastatic strategies that a tumor cells may utilize to successfully invade distant areas of the body [Shea 2002].

Introduction to Invasion Ecology and Metastasis

Ecology studies the dynamics of communities of species and their interactions, as well as their fitness effects on the interacting individuals. Fitness effects can be positive, negative or neutral. Tumor cells compete for space and nutrients (i.e. glucose, oxygen). Competition can have a negative effect within the same species (intraspecific competition), or among individuals of different species (interspecific competition). Limiting resources whose availabilities vary in time and space will select for dispersal [Merlo 2006]. For example, higher tumor cell density translates into more intense competition for space and nutrients and consequently movement and dispersal strategies.

In ecological interactions among individuals, predators can also control the population sizes of prey and select for anti-predator adaptations. Prey may evolve or adopt behaviors that tradeoff predation risk with foraging ability [Crespi 2005]. Predation also has a negative effect on individuals from different species. Predation can be observed within metastatic cancer through the interaction between tumor cells and cells of the immune system (see Chapter 5). Tumor cells have evolved numerous mechanisms for evading the immune system [Liu 2007; Krammer 2000].

Furthermore, the microenvironment of tumor cells may dramatically effect cancer progression [Kenny 2003]. In disseminated cancers, the seed and soil hypothesis suggests that metastasis is analogous to the colonization of a new habitat [Fidler 2003]. Some ecological studies have supported the hypothesis that increasing species complexity in an ecosystem facilitates the increased likelihood of outbound invasions [Shea 2002]. In an altered microenvironment, termed the “pathological niche”, it has been suggested that cells gain new genetic alterations leading to new functions and aptitudes. Such cells may drive tumor progression and permit invasion into new metastatic niches [Wels 2008]. Successful colonization depends on the conditions of the new environment including resource availability, competition from normal cells and predation from the immune system. A tumor can be viewed as a large population of heterogeneous cells competing for the same resources from an ecological and evolutionary perspective. Selection for dispersal and colonization likely depends on the heterogeneity of these tumor cells and a series of filters. Because each cancer patient is a novel evolutionary event, tumor cells cannot anticipate or be selected to metastasize. Rather, metastasis must be an unintended consequence and emergent property of the eco-evolutionary dynamics driving tumor cell evolution and diversification within the primary tumor.

The definition of an invasive species in ecology is an organism/species that is non-native to the environment, capable of surviving and flourishing in that environment, and is able to expand into sites beyond that of its initial establishment [Lockwood 2009]. Not all invasion events are successful, much like

the invasion events of a circulating tumor cell. It is the failures along the path of invasion that help us understand the full process of invasion as well as the qualities or characteristics that the invasive species may have that enable them to succeed. The stages of invasion are as follows: 1) transport, 2) colonization, 3) establishment, and 4) landscape spread [Theoharides 2007]. These stages are analogous to the steps in the metastatic cascade [Gatenby 2009]. The transport step requires species whose propagules can survive and be transported over long distances to a new region. The colonization step contains abiotic filters that determine whether propagules survive in the new habitat, and affect growth rates [Theoharides 2007]. The establishment step has several processes including 'biotic resistance' from the native species, and the suppression of the non-invasive species reproductive rates. The landscape-spread step indicates that spread rates of invasive species depend on establishment, dispersal ability, and habitat connectivity [Theoharides 2007].

There are failures within each of these steps that serve as filters for the invasive species. During transport, the invasive species could simply die or become depredated. During establishment, the invasive species might be outcompeted by the native species, may have limited access to nutrients, or may be immediately killed off. The result of any of these challenges is a failure to establish at the secondary site. During landscape spread, the invasive species may not have the opportunity to spread again; therefore it remains in the local region. Often invasive species are able to succeed because of a vacancy within the new niche, or the absence of predators and competitors. Furthermore, a change in the donor region can lead to a population increase of pre-existing resident species, such that more individuals would be available to interface with a transport mechanism (such as ballast water) or range expansion of local species into previously uninhabitable areas of the donor region, making these species available for transport [Carlton 1996].

Likewise, changes in the recipient region can lead to altered ecological, biological, chemical or physical states, thus increasing the susceptibility of the recipient region to invasion [Carlton 1996]. For example, altered water quality conditions lead to increased ability of pollution-intolerant or pollution-tolerant species

to invade. The release of a very large number of invasive species to the recipient region increases the number of introduction events, thus increasing the probability of a successful colonization and establishment. Alternatively, the environment of the donor or recipient regions may not change and still an invasion event could occur if the invasive species can survive the transport step.

All sites that receive new invasions thus become new potential donor regions. For example, the Laurentian Great Lakes are now exporters of the Eurasian zebra mussel, Dreissena. An analysis of in-ballast vessel traffic patterns departing these regions could provide insight into where these species may next appear [Carlton 1996]. A similar analysis could be applied to the study of metastatic cancer in the human body. Following the vasculature and blood flow patterns departing the infected organs could provide insight into where the next establishment may occur, given that circulating tumor cells (CTCs) occur in the blood. When an invasive plant takes over an area its root system may dominate, and can result in increased soil erosion [Belt 2009]. This can put local water resources at risk due to increased run-off. Such damage cannot be easily remedied, even after the invasive species are removed from the environment.

When a cancer cell establishes and develops a tumor in the secondary site, it may dominate that organ and disrupt the normal physiological and chemical processes of that organ, which places the native cells of the organ at a greater risk for survival. Reduced function in the “infected” tumor will then compromise all of the other organs that depend on its normal functions. Tumors are not easily eradicated from the body, even when surgically removed. To make matters worse, many cancer therapeutics are genotoxic, meaning that healthy tissue cells are being destroyed along with the tumor cells, providing a selective pressure for the proliferation of variant cells within those vacant spots and providing additional mutational insults that could improve the tumor cells’ fitness and malignant potential [Greaves 2012].

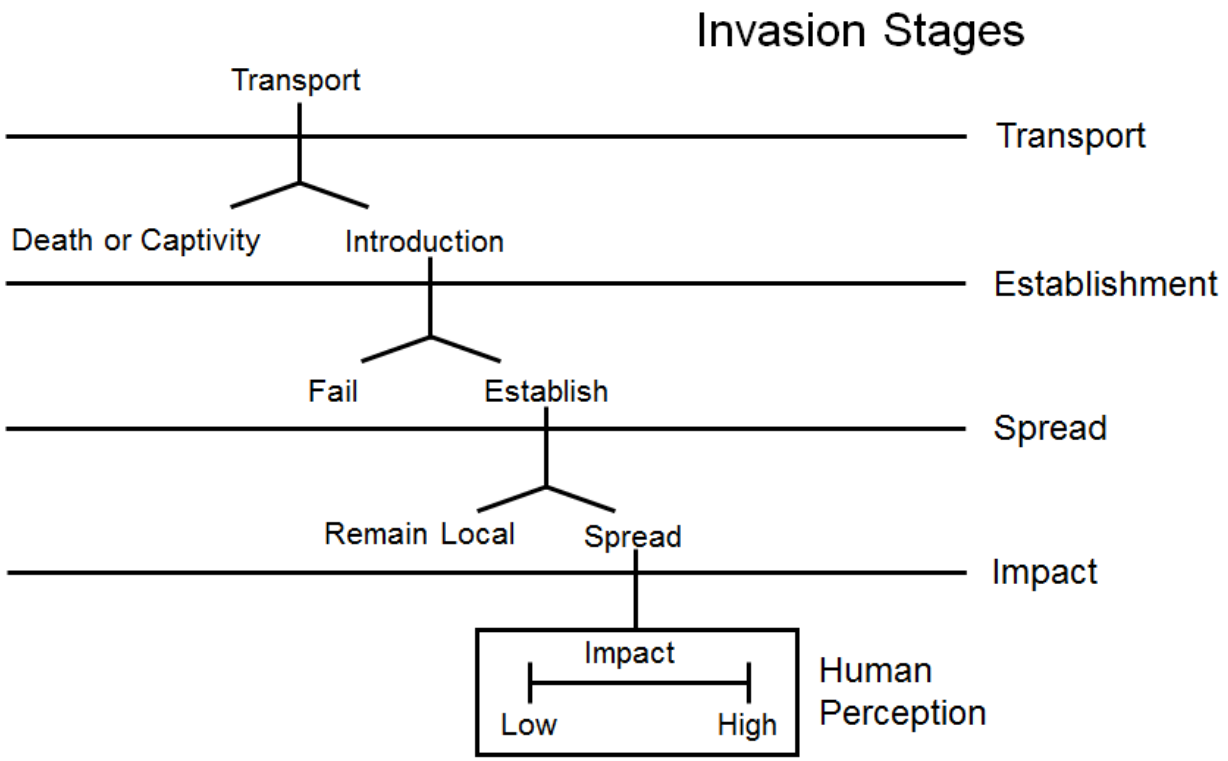

Figure 7.1 Recreated from Lockwood et al, 2007 to illustrate the invasion stages in ecology.

Concepts of Invasion Ecology and Metastasis

The principles of evolutionary ecology apply to cancer. Such principles can enhance our understanding of cancer processes and tumor dynamics [Merlo 2006]. Several ecological frameworks for cancer exist in the literature. For example, as presented by John W. Pepper, et al, the mathematical theory of Darwinian dynamics provides tools for understanding and predicting responses to somatic selection, including clonal adaptation, diversification, and extinction [Pepper 2009; Vincent 2005; Michor 2004]. This framework has been used to develop explanatory models of cancer initiation, promotion and progression [Vincent 2008; Lauren 2010]. Another example that uses the ecological framework was written by Merlo, et al (2006), examining how aneuploidy contributes to the evolutionary dynamics prevalent in neoplastic progression. They consider whether aneuploidy itself is selectively neutral or advantageous. Regardless, aneuploidy may act as a mechanism for accelerated mutation rates permitting more rapid adaptive evolution of cancer cells.

A neoplasm can be viewed from an evolutionary perspective as a large, morphologically, genetically and epigenetically heterogeneous population of individual cells [Lloyd 2015; Merlo 2006]. Comparative analysis of cancer cell phenotypes, genotypes, and phylogenies could be utilized to explain the molecular changes occurring during tumor progression to distinguish which tumor cell types give rise to the development of invasive, metastatic and resistant cell phenotypes. As stated in the article by Gatenby, Brown, and Vincent, "invasive populations are more likely to be successful when there are multiple introductions and if the originating population is genetically and phenotypically diverse" [Gatenby et al. 2009; Suarez 2008; Lavergne 2007]. Populations that do invade may pass through a genetic bottleneck in which the population is initially relatively genetically homogeneous [Chakrabory 1977]. Following such a bottleneck, successful invasive species typically undergo rapid evolution [Nei 1975]. Natural selection may favor an increased diversity of phenotypes and subpopulations with adaptations to exploit the opportunities and avoid the hazards of their novel environments [Lee 2002; Sakai 2003].

Merlo, Pepper, Reid, and Maley [Merlo et al. 2006] also review the evolution and ecology of neoplastic clones. They examine the consequences of these dynamics by identifying important missing pieces in the puzzle of tumor progression, prevention, treatment and what causes the progression to begin with. It is stated that the selection of individuals due to cancer has a significant impact on the developmental pattern of differentiated tumor cells and the suppression of somatic selection. Given these comparisons and ideas, we suggest that much can be gained by the application of the ecological and evolutionary principles to the development of cancer, its progression, and treatment plans.

It is important to recognize that there are several general hypothesis in invasion ecology. Here, for sake of completeness, we briefly review four which were identified by the authors as relevant to oncology and metastasis. Perhaps the most poignant connection between ecological invasion concepts and distantly metastasizing cancer cells is the 'enemy release' hypothesis [Colautii 2004]. As early as Thellung in 1915,

this concept is described as invasive success because of release from its natural enemies. It is plausible that cancer cells contend not only with the immune system but also with other cancer cells [Moreno-Smith 2010]. For this reason, successfully metastasizing to a distant site may permit a release from competition from other cancer cell populations and/or lowered exposure to any elevated immune response at the primary tumor.

Next, one might consider the propagule pressure hypothesis where an increased number and frequency of invasion events (in cancer, metastatic events) increases the likelihood of success [Colautti 2006; Lockwood 2005]. Namely, higher rates of release of circulating tumor cells from the primary tumor may increase the probability that a cancer cell will take hold and multiply in a distant.

The Tens Rule is similar in that approximately ten percent of a species' population takes a given step in the 4 step cascade of becoming an invasive species. Thus if 10% of a species are transported, 10% of those colonize, 10% of those become established and 10% of those spread in the landscape [Lockwood 2001; Jeschke 2014], then at most 1 in 10,000 individuals will give rise to a successful invasion. In cancer one might suggest proposing at least one order of magnitude less at each stage, and metastatic cancer cells may not support the general linearity of this 'rule of thumb'. A 1% rule poses interesting estimates- tumors are often made up of billions of cells, if 100 million cells intravasated and 1 million could successfully circulate then 10 thousand might extravasate and 100 might establish. This could be reasonable. This is pure conjecture but is an interesting thought-exercise and an interesting hypothesis to investigate further.

Finally, the last hypothesis for invasion ecology that we will discuss is the Biotic Resistance hypothesis [Rius 2014]. Here, the greater the biodiversity of the recipient ecosystem, the greater its ability to resist invasion. This is a well-studied hypothesis in ecology, yet it is unclear that this hypothesis readily applies to cancer. Yet, by fact, perhaps this is why metastasis is such a difficult (yet deadly) feat for cancer cells to achieve. There is at least some evidence that one thousand to one million CTCs are present in about

20% of all breast cancer patients, yet very, very few actually succeed [Rack 2014]. Perhaps this is due in part to the body's diverse cell types. This is an area in which a great deal more investigation is merited.

As previously mentioned, the success of an individual tumor cell resulting in a metastatic event is infinitesimal [Merlo 2006]. The success of invasive species, such as tumor cells, heavily depends on a series of filters. The important filters that accompany the stages of metastases may be dispersal-limitation, recipient-limitation, organ-specific signaling, number of circulatory rounds before arrest, or nutrient-limitation [Fidler 2003].

Filtering Concepts as Limits to Metastatic Success

Allele frequencies can change (evolution can occur) through dispersal [Merlo 2006]. Dispersal theory suggests that high cell mortality and variation of resources and population densities across space might select for metastasis. Separating invasion into stages allows us to compare patterns of non-indigenous invasive species success from disparate studies and to discuss the relative importance of filters to invasion at each stage [Theoharides 2007]. Further, identifying the stage at which an invasion fails may allow us to understand the interaction of invasion filters with invasion character (e.g. number of introduction events), species traits and strategies and ecosystem characteristics. Individual tumors may be comprised of diverse "species" of clonal lineages that occupy distinct "niches". Among these species, subpopulations may exist that contain metastatic properties which facilitate colonization of secondary sites and lead to poor patient prognosis.

As mentioned before, the success of invasion depends heavily upon a series of filters. The first filter, dispersal limitation, refers to the ability of certain tumor cell types to intravasate the blood vasculature and survival within the vasculature. Dispersal theory suggests that high cell mortality and variation of resources and population densities across space might select for metastasis [Merlo 2006]. There are at least three ways in which dispersal can be important in cancer: the movement of cells between the partially isolated sub-populations of proliferative units, local invasion of neighboring tissues and emigration of metastatic

cells from the primary tumor. Whether dispersal distance is declared to be short or long will largely depend on the system and particular colonizer under scrutiny [Showalter 2008]. Nonetheless, short-distance dispersal is considered to be primarily between adjacent, or nearly adjacent, environments. On the other hand, long-distance dispersal can be viewed as movement typically between widely distant environments, usually separated by a barrier of some sort, a process that could be termed saltation dispersal or punctuated dispersal [Showalter 2008]. Note that subsequent short-distance dispersal originating from within the newly colonized environment may often follow an initial colonization episode precipitated by punctuated dispersal.

Recipient limitation and nutrient limitation both refer to the ability for metastatic cells to extravasate from the vasculature system into a new secondary site and survive within the secondary site. In oncology this may be considered organ-specific limitations. It is known that certain organs of the body receive the most invasive cancer cells while other organs shed/disperse the most cancer cells [Scott 2012]. In ecology, the same patterns are observed. Certain types of habitats seem to have higher numbers of established non-indigenous species than others. For example, islands are more vulnerable to invasion because they usually have fewer resident species to begin with, leading to the conjecture that simpler systems have less biotic resistance to invaders [Elton 1958; Brown 1989]. Larger islands can support more species and have lower extinction rates than small ones because they cover larger areas, with a greater diversity of resources and habitats [Robinson 1989]. Less isolated islands tend to support more species than remote ones, because they have higher rates of immigration. The same ideas can be reflected on the organs of the body. For example, the brain could be considered a large isolated island that is seen to receive many invasive cancer cells, yet seldom releases tumor cells (Table 7.1).

Metastatic breast cancer accounts for 20 percent to 30 percent of the 170,000 cases of brain metastases diagnosed annually, and as improvements in systemic therapy prolong survival, brain metastasis in breast cancer patients is becoming more evident [Vallow 2007]. Estimates suggest that 35 percent of lung cancers, 10 to 30 percent of breast cancers, 30 to 40 percent of melanomas, five to 10 percent of kidney cancers, and five percent of colon cancers metastasize to the brain (Table 7.1) [Appendix 1; reference 15].

The liver is another organ that could be considered as a large island abundant in resources and nutrients, making it vulnerable to invasive species. The liver is a common site for metastasis in almost all types of tumors. The liver is the third most common site for the spread of breast cancer after the bone and lung (Table 7.1) [Appendix 1; reference 12]. Studies show that the liver is the first site of metastases for disseminated breast cancer in approximately 10 percent of cases (Table 7.1) [Appendix 1; reference 12]. Two-thirds of women with metastatic breast cancer eventually manifest a spread to the liver (Table 7.1) [Appendix 1; reference 12]. Liver metastases develop in approximately 60 percent of patients with colorectal cancer (Table 7.1) [Appendix 1; reference 9]. In fact, the liver is the only site of metastasis in up to 35 percent of patients with metastatic colorectal cancer.

There is a fair amount of reciprocity shown in lung and melanoma cancer, on the other hand. In Table 7.1, it is apparent that lung and melanoma will spread to all the regions listed, while also receiving metastatic cells from other primary tumors in a few of those regions. Second to the bones, the lungs are a primary site for breast cancer spread (Table 7.1) [Appendix 1; reference 4]. Studies show that the first metastases go to the lungs in approximately 19 percent of cases. Lung cancer is the most common cancer that spreads to the brain, and at least 40% of people with advanced lung cancer will develop brain metastases sometime during their disease (Table 7.1) [Appendix 1; reference 19].

	Breast	Liver	Prostate	Lung	Glioma	Pancreatic	Ovarian	Colon	Renal	Bone	Melanoma
Breast	NF	NF	0.4-0.45% [58]	< 3% [17]	0	NF	1-3% [2]	NF	NF	NF	70-82.6% [24] or 40% [25]
Liver	6% [45] or 1029-13% [33] or 5.2% [29]	NF	~25% [10, 11, 30]	33-40% [43]; SCLC 17.5% [18]; NSCLC 3.8% [18]	0	50% [57]	55% [35]	60-70% [9, 12, 13] or 10-20% [29] or 40-70% [13, 14]	20-40% [42] or 30-40% [54]	NF	50% [16] or 54-77% [41]
Prostate	NF	NF	81% [33]	NF	0	NF	NA	NF	NF	NF	NF
Lung	60-70% [3] or 19% [4] or 17% [45]	37- 44% [22]	50% [11]	NF	0	NF	22% [35]	10-20% [4]	50-60% [54] or 72-76% [27] or 32% [28]	50% [50]	12% [4] or 70-87% [24,25,41]
Glioma	6% [45] or 10-16% [5] or 10-30% [15] or 20-30% [7]	1.3-2.9% [20,21]	1-2% [11] or 7.5% [31]	18 - 65% [6] or ~40% [19] or 15-43% [43] or 35% [15]	NF	NF	.29-11.6% [36]	5% [15,54]	5-10% [15] or 13.2% [26]	4.7% [51]	30-40% [15] or 44% [23,26] or 36-54% [41]
Pancreatic	<3% [46]	~2% [32]	1.4% [31]	<3% or 30% [44]	0	NF	~2% [32]	<1% [40]	12-26% [47]	~2% [57]	38-53% [41]
Ovarian	~40% [1]	NF	NA	0 - <1% [59]	0	NF	75% [1]	381-6% [37] or 5 10% [38]	NF	NF	NF
Colon	~45% [49] or 3% [55]	NF	NF	NF	0	NF	0	NF	<1% [53]	NF	26-58% [41]
Renal	NF	NF	3.1% [31]	<3% [60] or 16-23% [43]	0	NF	50% [52]	NF	NF	NF	35-48% [41]
Bone	51% [45] or 70% [48]	NF	90% [16,31] or 60% [47]	14-40% [8] or 19-33% [43] or 30-40% [19,20] or 80% [4]	0	5-20% [31]	0.1-0.12% [34]	16-34% [39]	30-40% [54]	NF	23-49% [41]
Melanoma	30% [56]	NF	0.7% [24]	4% [56]	0	NF	<1% [35]	9% [56]	NF	NF	65-70% [41] or 45% [56]

Table 7.1 Metastases matrix model showing the amount of metastatic incidences as percentages in eleven major organs. The chart is read from top to bottom and left to right, where the top row indicates the primary site of tumor growth and the column on the left indicates the secondary site of metastasis. NF indicates that information was not found. References for Table 7.1 can be found in APPENDIX 1.

Although the matrix of metastases between primary and secondary tumors shown in Figure 7.1 contains a few areas where information is lacking, reciprocity or asymmetry of metastasis between the organs could still be clearly seen, and estimates could still be made. Out of the ten major organs reviewed, the ovaries, prostate, breasts, colon and kidneys (renal) are seen to be the largest donor organs. While the liver, brain (glioma), bone, lung, pancreas and skin (melanoma) are seen to be the largest recipient organs. Here we examine another hypothesis that examines metastasis as a clustering vs. nesting process, where clustering represents sets of organs (or pairs of organs) that reciprocally metastasize within the cluster (e.g. digestive system organs, reproductive system organs, respiratory system organs, etc) but do not metastasize nor receive metastases from organs outside of the cluster. Nesting would represent a perfect set of asymmetries, where the most common donor metastasizes everywhere, then the next most common donor metastasizes to all the other organs except for the one it received metastasis from, and so on. In Table 7.2, one can also see evidence that the main sites of metastases from primary cancers are the bone, liver, lung, brain, and although not included, the pancreas is a frequent site for metastases as well. Therefore, we conclude that the general metastatic cascade of cancer is an overall asymmetrical nested process.

Cancer type	Main sites of metastasis*
Bladder	Bone, liver, lung
Breast	Bone, brain, liver, lung
Colorectal	Liver, lung, peritoneum
Kidney	Adrenal gland, bone, brain, liver, lung
Lung	Adrenal gland, bone, brain, liver, other lung
Melanoma	Bone, brain, liver, lung, skin/muscle
Ovary	Liver, lung, peritoneum
Pancreas	Liver, lung, peritoneum
Prostate	Adrenal gland, bone, liver, lung
Stomach	Liver, lung, peritoneum
Thyroid	Bone, liver, lung
Uterus	Bone, liver, lung, peritoneum, vagina

Table 7.2 Major site of metastasis as recreated from National Cancer Institute: metastatic cancer fact sheet.

We predict that the reason such organs are the main sites of metastases is because their normal tissue density is medium to low and the amount of nutrients available is very high (high vasculature to normal cell density ratio). This may create favorable conditions for a metastatic tumor cell. Also, because these areas are generally highly vascularized, the cell density per vessel is not very high, thereby allowing vacant niches for the tumor cells with abundant access to space and nutrients. Donor organs are considered more of the 'desert' type of organs where the normal cell density is very high and very limited nutrients are available, thereby allowing the tumor cells to evolve selection for dispersal to nutrient rich organs. The high cell density allows more tumor cells to be sloughed off, if near a blood vessel or angiogenic vessel. Blood flow within regions of tumors typically results in temporal and spatial variations in concentrations of growth factors, substrate, and metabolite concentrations [Lloyd 2014]. Each of these variations represents selection forces that could drive the adaptive evolution of local tumor populations.

Potential Strategies Leading to Metastatic Success

The development of a nomenclature scheme based on types of tumor cell colonizers might clarify communication within the fields of both invasion ecology and oncology [Showalter 2008]. Here I suggest distinguishing the tumor cell traits into five distinct strategies: the ecological engineer strategy, the ecological pioneer strategy, the penguin theory, the treasure-seeker strategy, and the canoeist strategy (Figure 7.2). These five tumor cell strategies provide possible explanations for the cancer's ability to overcome several of the different filters represented in each of the four stages of metastasis.

Given the struggle for cancer cells to survive in the face of biotic and abiotic pressures, five metastatic strategies have been suggested:

- Ecological Engineers build the infrastructure they need to proliferate
- Ecological Pioneers mobilize to seek new resources
- Penguins are 'forced off their ice float' by intratumoral pressures
- Treasure Seekers intentionally migrate following a nutrient gradient
- Canoeists intentionally utilize vascularization as a mode of transportation

Figure 7.2 List of five (5) proposed metastatic strategies discussed.

The traits of cancer cells likely influence transport. The traits of these five cancer cell types may have pre-adaptations for motility and survival. The “ecological engineers” and “penguin” cells represent this dispersal limitation and recipient limitation because they do not acquire the capabilities for dispersal since they are non-motile; they have no pre-adaptations for survival in the blood and are not able to extravasate on their own. This limits their reaching areas of suitable habitat. The “pioneer” and “ecological engineer” cells, however, are much more successful at co-opting a new environment than the “penguin cells” when they happen to finally land onto a new environment, which in this case would be the secondary site of metastasis. Variability in resources within a tumor and primary organ is almost always the driving factor for selection of cells with high motility [Chen 2011].

The “treasure-seekers” and “canoeist” represent the recipient limitation because although they are excellent at intravasation and survival of the blood stream, they cannot extravasate and colonize the secondary site as easily. As described in Table 7.3, the “canoeist” cells are only capable of colonizing a new environment with a pre-existing tumor mass because they usually only represent short-range dispersal, probably due to competition for resources; it will use the tumor’s weaknesses to its advantage and exploit all the nutrients and resources within that tumor and then move on to another site, using the blood vessel network as a means of transportation from point A to point B. Species that pass through the transport phase do not necessarily colonize their destination area [Theoharides 2007]. “Survival depends on environmental conditions (e.g. soil type and climate) and biotic processes at the neighborhood scale. Arriving populations must survive and achieve positive growth rates at low densities“ [Chesson 2000; Sakai et al. 2001].

	Ecological Engineer Strategy	Penguin Theory	Ecological Pioneer Strategy	Treasure-Seeker Strategy	Canoeist Strategy
Niche	Interior of tumor with access to blood supply	Interior of tumor near the edge of angiogenic blood vessels	Edge of tumor	Edge of tumor	Edge of blood vessels
Mechanism	Unintentional migration; Pushed into blood stream from intra-tumoral pressure; Interactions with mesenchymal cells that govern growth and function of new blood vessels and construction of extracellular membrane	Unintentional migration; Pushed into blood stream from intra-tumoral pressure	Unintentional migration; Pushed into blood stream from intra-tumoral pressure; Maximizes proliferation; Maximizes growth of competitors	Intentional migration; Follows nutrient gradient; Searches for space	Utilizes bloodstream as a means of transportation; intentional migration; follows nutrient gradient; searches for space
Pre-adaptations	Has ecological tools for building a favorable environment in secondary site; Promotes tumor cell proliferation by interaction with mesenchyme	Has access to nutrients and space	Adapted to co-opt in new environment; Has ecological tools and survival strategies within primary tumor, blood and secondary sites; Optimizes invasive characteristics	Motile; Can migrate toward nutrient-rich areas; can extravasate; can travel long distances	Motile; Can "swim" and survive blood stream; More likely to explore new environments; Can extravasate
Handicaps	Not pre-adapted for survival in blood stream; cannot extravasate; not motile; Limited survival in blood stream; Increases number of mesenchymal cells, provides competition and reduces number of tumor cells	Not pre-adapted for survival in blood stream; Not motile; Can't "swim" or extravasate; Limited survival in blood stream	Very destructive; Cannot promote its own mesenchyma; Must remain in continuous motion; Resources become exhausted and local infrastructure destroyed due to no interaction with host mesenchyma	Cannot survive in crowded area; Seeking nutrients leads to getting "pushed in"	Not pre-adapted for survival in secondary site with no pre-existing tumor

Table 7.3 Overview of the five tumor cell metastasis strategies and the perceived niche, mechanism of metastasis, pre-adaptations to a novel environment and potential handicaps.

“Treasure-seekers” on the other hand are also capable of intravasation and surviving the blood stream, however, their intravasation is not always intentional. They exploit the nutrients within their tumor mass, and at times their quest for nutrients and space leads them to the edge of the tumor where they are forced into the blood stream from the intra-tumoral pressure exerted upon them. These “treasure-seekers” are able to travel long distances. Thus, they may circulate around the body many times and may never be able

to colonize. Once they do have the ability to colonize a secondary site, however, they can be very destructive and competitive.

Colonizing individuals often have high fitness because they can escape from deteriorating local conditions caused by population growth and the over-consumption of resources. "The high density of an organ and the neoplasm present inside that organ suggest that space and nutrients are limited. This leads to fierce competition, so there might be selection for dispersal" [Merlo 2006]. Again, the selection for dispersal refers to short-range dispersal within the tumor itself or among neighboring tissues. These cancer cell types will not be selected for dispersal through the bloodstream because of the overwhelming risk for survival within the bloodstream.

"Ecological engineers", "ecological pioneers", "treasure-seekers", and "canoeists" would be the cancer cell types that would be the most successful at colonizing a secondary site upon arrival. Once these cells begin to establish in their new environment, biotic filters come into play and will further select for the most adapted and successful cell type. Biotic filters are barriers to invasion created by the actions or presence of living organisms. This is also known as the seed-soil interaction. Similarly in invasion ecology, organisms that are capable of colonizing islands naturally are typically adapted to be dispersers (such as the "pioneers", "treasure-seekers" and "canoeists"), are small, and numerous in their original habitat and have a propagule that can be dispersed over water, through wind or vectored by an animal from the mainland [Colautti 2006]. Thus, identification of tumor heterogeneity and distinctive environmental regions are necessary for understanding the roles of each cell within its environment and finding clinical treatments that match these characteristics.

We hypothesis on the types of cancer cells found in the eleven major organs subject to cancer. In the breasts, they are most likely "penguin" tumor cells. This is because the neoplasm itself is so densely packed that a high number of tumor cells are bound to be unintentionally pushed into the vasculature (Table 7.4).

The breast could also contain “ecological engineers” that will construct angiogenic vessels within the tumor to gain access to more nutrients. They also might be unintentionally pushed into their own angiogenic vessels due to the high density of the tumor. Since there are angiogenic vessels, you may expect to find the “treasure seekers” and the “canoeists” that will intentionally intravasate from the primary tumor and use chemotaxic signaling and response to colonize another site that is richer in nutrients. The “pioneers” would probably be the most common type of tumor cells that leave the tumor and colonize another organ. These might be the most destructive metastatic cells.

	“Ecological Engineers”	“Penguin” Theory	“Pioneer” Theory	“Treasure Seeker” Theory	“Canoeist” Theory
Breast	4	5	2-3	3	3
Liver	2	2	3	5	2
Prostate	3	2	4	4	2
Lung	3	5	2-3	2	4
Glioma	1	1	4	5	4
Pancreatic	2	2	3	5	2
Ovarian	4	5	3	3	3
Colon	2	3	4	4	2
Renal	1	2	4	5	3
Bone	4	3	5	2	4
Melanoma	2	2	4	5	3

Table 7.4 Cell types by cancer origin. The numbers indicate the probability of types of tumor cell occurrence in each organ. 1 = lowest probability, 5 = highest probability

The liver would most likely contain the highest number of “treasure seeker” tumor cells compared to the other types of cells, since the liver is nutrient rich and the cells are not as densely packed, which is also why there is a low probability of having many “penguin” cells. The “pioneer” cells and “treasure seeker” cells have the highest probability of occurring in the prostate because the environment is low in nutrient availability and space. Therefore, there might be selection for dispersal among the tumor cells within the neoplasm in the prostate.

In the lung, “penguin” cells may have the highest probability of occurring because the tumors are so densely packed and ample vascularization is available. This provides ample opportunity for unintentional migrants

as cells get pushed into the blood stream. A decent amount of “canoeist” cells might be observed as well because they are able to migrate throughout the tumor and create more metastatic tumor masses within the lung, and can migrate from one lung to the other, following any nutrient gradients. Since the lung already contains many capillary beds and venules, it is assumed that there would not be much need for the construction of angiogenic vessels; therefore selection would not favor “ecological engineers”. But in cases when the tumor cells are so densely packed within the neoplasm, the cells around the necrotic center would seek access to nutrients and space, and this could provide selection for some form of “ecological engineer” cells. Now, since the lung is also a highly nutrient rich organ, the tumor cells would not have to travel far to reach areas rich in nutrients and so selection may not favor “treasure seeker” cells. The “pioneers” have about the same probability of occurring as the “ecological engineers” because they would be the types of cells occupying the edge of the tumor that get unintentionally pushed into the vasculature. However, once they are inside the vasculature, they are able to utilize their pre-adaptive survival strategies to extravasate onto a new environment and optimize invasive characteristics. These “pioneer” cells would be the metastatic cells.

While spectulative, we hypothesize that, the brain may receive a lot of “treasure seeker” cells because the environment is high in nutrients and the normal cell density is low. Since the cell density is low, it is expected that there may not select for many “ecological engineers” or “penguins”. The next highest occurrence of cells to be observed would probably be the “canoeists” and “pioneers” because these cells have motility ability, moving from point A to point B via the vasculature to obtain nutrients, explore new environments, and optimize invasive characteristics.

In bones, the “pioneers” have the highest probability of occurring because the bone is extremely nutrient rich such as the lungs and brain, so the tumor cells would have to be in constant motion to try and access as much nutrients as possible. “Ecological engineer” cells and “canoeist” cells would also be selected for because of the need to create angiogenic vessels within the tumor to gain access of more nutrients and be able to migrate from bone to bone. Space availability is ample within the bones and so densely packed

tumors may not be an issue here, which is why there might be little to no “penguin” cells available. The kidneys, much like the lungs, will most likely contain the “treasure seekers” because the kidney is a nutrient rich environment for the tumor cells. The next highest occurrence of tumor cells would be assumed to be the “pioneers” because those would be the metastatic cells that are unintentionally pushed into the vasculature, and are able to survive within the blood stream. The “canoeists” have a moderate probability of occurring because they can migrate well within the kidney tissue. These are intentional migrants and possible metastatic cells as well; they move toward the nutrient gradient and space. Since there is abundant vasculature within the kidney, not many “ecological engineers” would be selected for, except in the center of densely packed tumors.

The pancreas, much like the brain, would receive the most “treasure seeker” cells and contain a fair amount of “pioneers” because it is a nutrient rich environment. The ovaries are assumed to have “penguin” cells as the highest probability of occurrence because the cells are so densely packed that not a lot of space is available and due to the intratumoral pressure exerted upon the cells at the edge of the tumor, many of them get pushed into the vasculature. Therefore, it is assumed that many “ecological engineer” cells would be selected for as well, because the densely packed tumor cells need to create angiogenic vessels to gain access to nutrients.

The colon would contain the highest amount of “treasure seeker” and “pioneer” cells because the colon is not a very nutrient rich environment, therefore many of the cells may intentionally migrate to an area that is more suitable for growth. The skin also has a high amount of “treasure seeker” and “pioneer” cells. The skin is unique in the fact that it may disperse and receive many of these cells along with the rest of the organs mentioned above. The skin is actually considered a nutrient poor environment because of its constant exposure to the harsh environment of the air. The skin may receive its nutrients through diffusion from the blood vessels underlying the surface. Therefore, intentional migrant cells would be the most abundant because they must remain in constant motion to seek out proper nutrients for survival and growth. Knowing the types of the tumors that could be involved in these metastatic cascades within each major

organ that is susceptible to metastasis can provide significant clinical value for the development of treatments.

While this section is mostly conjecture and is highly speculative, and meant to encourage investigations and thoughts about the traits associated with metastasis. The idea being that selection within a tumor creates a diversity of “species”, some of which are more likely to occur and metastasize than others in predictable patterns.

Conclusion

In order to understand cancer, we must first begin to understand the population dynamics and evolutionary parameters of neoplasms. Understanding cancer through the lens of evolutionary ecology helps us grasp the reasons behind why certain metastatic processes occur the way they do, what controls them, and how to potentially stop them from occurring. Invasion ecology can be used to understand biological invasions by applying niche concepts, comparing the two processes to one another, and evaluating species heterogeneity. These ideas lead to the study of tumor cell heterogeneity and the types of communities (organs) that they invade. Tumor cell heterogeneity continues to further study because knowing specifically what types of cancer cells invade which areas in the body and how this may provide a greater understanding for the development of new treatments. We have concluded that metastases is an asymmetric nesting process and that there seems to be five major types of tumor cells that are involved in the metastatic cascade: “penguin”, “ecological engineer”, “treasure-seeker”, “canoeist”, and “pioneer”.

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APPENDIX 1

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VITA

Mark Cassidy Cridlin Lloyd
February 19, 2016

Current Position: Senior Scientist, Executive Vice President and Founder
Inspirata, Inc.
1 North Dale Mabry Highway
Tampa, FL 33609
(813)-570-8910
(813)-541-1238
Mlloyd8@uic.edu

Education:

1996 – 2000:	Dickinson College, Carlisle, PA
	Bachelors of Science
	Biochemistry and Molecular biology and Biology
2006 – 2008:	University of South Florida, Tampa, FL
	Masters of Science of Management

Academic and Professional Experience:

2000-2002	Research Assistant II, Lombardi Cancer Center, Georgetown University, Washington, DC
2002-2003	Molecular Pathology Assistant, Lombardi Cancer Center, Georgetown University, Washington, DC
2003-2005	Research Associate, Analytic Microscopy, H Lee Moffitt Cancer Center and Research Institute, Tampa, FL
2005-2006	Supervisor, Analytic Microscopy, H Lee Moffitt Cancer Center and Research Institute, Tampa, FL
2006-2014	Staff Scientist I, Analytic Microscopy, H Lee Moffitt Cancer Center and Research Institute, Tampa, FL
2012-present	Lead Scientist, Executive Vice President and Founder; Inspirata, Inc., Tampa, FL

Honors, Awards and Professional Societies

2015	Govenor's Business Ambassador Award
2014	Research Achievement Award; University of Illinois at Chicago
2013	Teaching Award; University of Illinois at Chicago
2001	Lombardi Cancer Center Award of Excellence
2007	Moffitt Research Team of the Year Award
2012	Best Scientific Poster; NCI/PSOC Annual Conference
2007	National Honors Society
2008	Phi Kappa Phi- Graduate Honors Society
2008	Beta Sigma Gamma- Business Honors Society
2008-present	American Management Association
2008-present	National Council of University Research Administrators
2008-present	SRA International
2006-present	American Association for Cancer Research
2003-present	Microscopy Society of America
1999-2008	Southern Society for Microscopy

Patents

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