Minireview

The great escape: How metastases of melanoma, and other carcinomas, avoid elimination

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Impact statement

Cancers kill mainly because metastatic disease is resistant to systemic therapies. It was hoped that newer targeted and immunomodulatory interventions could overcome these issues. However, recent findings point to a generalized resistance to elimination imparted by both cancer-intrinsic and -extrinsic changes to provide survival advantages to the disseminated tumor cells. Here, we present a novel conceptual framework for the microenvironmental inputs and changes that contribute to this generalized therapeutic resistance. In addition we address the issues of experimental systems in terms of studying this phenomenon with their advantages and limitations. This is meant to spur studies into this critical aspect of tumor progression that directly leads to cancer mortality.

Abstract

Cancer mortality ensues from metastatic growths. Cancers use two strategies to allow for this unrelenting expansion. The first way is that early metastases are often cryptic or dormant, being invisible to both innate suppressive actions and undetected clinically. Second, both the micrometastases and later clinically lethal growths are resistant to therapies, whether standard chemotherapies, targeted biologics, or even immunotherapies. These two modes of resistance necessitate new approaches to treatments if we are to eliminate mortality from solid tumors. However, to develop such therapeutic strategies, we first need to better understand the cellular behaviors and molecular events that enable the resistances. Herein, we present a comprehensive model of changing methods of avoidance and resistance that occur during tumor progression, and doubly confound treatment by mixing survival strategies throughout the continuum creating moving targets. Melanoma is presented as the model cancer, as it is being targeted by all three types of agents for disseminated disease, with breast and prostate cancer as two other key carcinomas.

Keywords: Liver metastasis, metastatic models, tumor microenvironment, hepatic niche, microphysiological

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Introduction

Metastatic progression of solid tumors is the harbinger of most morbidity and mortality from solid tumors. The initial primary tumor growth is most always capable of being eliminated by surgical excision or radiological ablation. While this may lead to real morbidity from the surgery and its sequelae, and the unusual death, the outcomes are increasingly curative as we appear to catch many at their earliest stages. Over 80% of melanomas and breast and prostate cancers are eliminated in this manner. However, if the tumor has already disseminated, the long-term outlook becomes bleaker with 5- and 10-year survivals falling dramatically; for melanoma, fewer than a third of patients survive past a half dozen years.

The issues that confront the survivors of the primary lesions are that the metastases are not only not amenable to removal, due to wide dissemination, but also that these cancer cells are generally more resistant to cancer-targeting agents. This is seen most clearly with neo-adjuvant breast cancer treatments that will shrink the breast nodule, even leading to a complete pathological response (no cancer cells detectable) upon subsequent lumpectomy, while not significantly impacting the overall survival.[1–3] At least a subset of the disseminated cells present distinct properties from the primary tumor cells in terms of therapeutic responsiveness.

As this is seen even with therapy naïve cancers, it is unlikely that this is due to genetic mutations, but rather is microenvironmentally imprinted on these cells in ectopic sites.

To overcome such resistance to therapy, we need to understand the phenotypic and cellular changes that cells undergo during progression. Initial studies have begun to dissect such switches and decipher their impacts on therapeutic responsiveness. Herein, we propose a unified model for tumor progression linking the microenvironmental induction of phenotypic switches with susceptibility to therapeutic approaches. This provides for testable hypotheses and pathways to re-sensitize disseminated tumor cells to accepted therapies.

The metastatic cascade

Dissemination from the primary site relies on the ability of tumor cells to survive and grow in a metastatic site and requires many changes to adapt to the tissue-specific environment unique to each organ.[4] Tumor metastasis, even more than carcinogenesis, involves both the carcinoma cell and the host environment (Figure 1).

Dissemination most likely requires distinct cancer phenotypic switchings. Initially, a cancer-associated epithelial-mesenchymal transition-like plasticity (cEMT) allows for the autocrine signaling and loss of cell–cell constraints that promotes escape from the primary site.[6,7] It should be noted that in referring to cells as epithelial or mesenchymal, we are denoting the presence or absence of cell surface E-cadherin, loss of cell–cell cohesion, and shape shift towards fibroblastoid without epithelial cell polarity; this is not a true phenotypic switch at the full transcriptome level.[8] This phenotypic plasticity, in part driven by the now-enabled autocrine growth factor signaling through the EGF receptor[9,10] and downstream involving the Met receptor for HGF.[11] This is further augmented by signaling from an altered localized microenvironment, both the matrix and the cells. This resembles a wound environment, with activated fibroblasts (CAF, cancer-associated fibroblasts) and an immature reparative matrix.[12–15] The productive fibroblasts secrete signals not only for the cancer cells but also to attract immune cells that further promote invasion and dissemination.[16,17] The combination of a mesenchymal, highly motile and physically plastic cancer cells with a disrupted pliant matrix allows for invasion through these barriers, whether the limiting basement membranes and outer muscular capsules in carcinomas or the thicker dermis in melanomas.

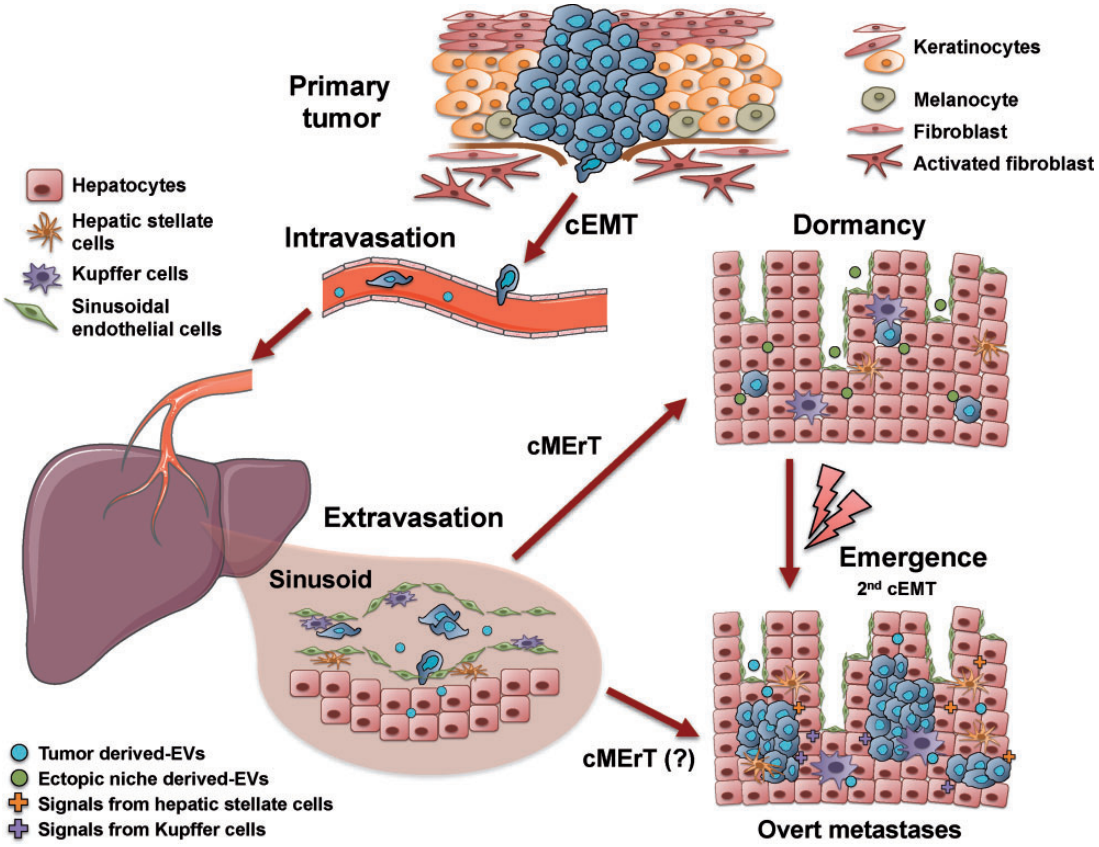

Figure 1. Metastatic cascade to the liver. Schematic of metastasis with postulated phenotypes that confer resistance or sensitivity to therapies. Disseminating carcinoma cells must acquire mesenchymal-like migratory properties to escape the primary locale (the cEMT). Transit through vascular conduits to sites of metastasis are fraught with challenges with most tumor cells not surviving, but are sufficiently transient to not represent a target for therapy. At the metastatic site, cells must survive apoptotic cytokines initially and chemotherapy later; this is accomplished by a reversion to a more epithelial phenotype and expression of E-cadherin (cMErT) that in turn provides for the chemoresistance and immune silence (PD-L1-negative), driven at least in part by host organ extracellular vesicles (green bubbles). Cells emerge from dormancy due to inflammatory stimuli (lightning bolts) to form aggressive, lethal metastatic nodules with re-acquisition of mesenchymal-like behaviors and cause the organ to secrete factors with secondary metastases also ensuing (adapted from Clark et al[5]). (A color version of this figure is available in the online journal.)

After transiting these barriers, the cancer cells must survive travel in a flowing conduit to metastasize. Whether the intravasation occurs in the hematogenous or lymph vasculature, the final extravasation is occurs from the hematogenous capillary bed (lymph node seeding is a separate category of dissemination from ectopic organ metastasis). While the processes of this transit and survival in a turbulent flow are complex, this short event is neither a reservoir of tumor cells nor an opportunity for treatment, and will not be discussed further.

At the other end of dissemination, the greatest challenge for metastatic seeding is integration into the ectopic metastatic tumor microenvironment despite the lack of a supportive environment and presence of pro-death signals from a local inflammatory response. Interestingly, metastases of breast and other carcinomas often express E-cadherin,[10,18–20] whose initial loss is the hallmark of cEMT and correlates strongly with dissemination,[21] even if expression of mesenchymal markers are absent.[22] This reversion to an epithelial phenotype is secondary to loss of mechanisms that suppress E-cadherin expression such as loss of promoter methylation[18] and Kaiso binding to the promoter,[23–25] and abrogation of EGF receptor signaling that downregulates E-cadherin.[9] We have reported that carcinomas and melanomas re-express E-cadherin in metastatic tissues such as liver, lung and brain,[8] while others have reported such in bone marrow metastases,[19,20,26] but not in lymph nodes (data not shown). Other epithelial markers including connexins are similarly upregulated in the metastatic niche.[21] This reversion of cEMT or a cancer-associated mesenchymal-epithelial (reverting) transition (cMErT) is only partial to the same extent as the initial cEMT is also partial,[8] suggesting a metastable or plastic situation that allows for outgrowth upon a second cEMT.[7]

A portion of these disseminated cells can enter a period of dormancy to later emerge as aggressive metastatic tumors. This 'dormancy' may be quite transient, as in the case of triple negative breast cancers, or extended as in hormone receptor-positive breast cancers (even in excess of two decades before clinically evident recurrence). However, evidence is accumulating that epithelial reversion is a feature of dissemination for most all carcinomas and likely melanomas. The mechanisms underlying dormancy are poorly understood as successful metastatic seeding and dormancy (as opposed to primary escape and dissemination[27]) are rare events.[28,29] This gap in our understanding is due in large part to the absence of tractable experimental systems that can focus on this stage to metastasis, a limitation we will discuss later. Successful ectopic seeding with its epithelial reversion appears to be linked with entry into dormancy. The tumor signals that enable intercalation within a tissue (including extracellular vesicles (EV))[30–33] leads to reverse signaling (including EV) from the receptive organ that imparts the cMErT and initial dormancy.[34,35]

The state of the tumor cell in dormancy is unknown, with one model positing quiescence versus another of balanced proliferation and death. *In silico* modeling determined that it is highly unlikely that micrometastases exist in a state of balanced proliferation and death, but rather either grow out or enter quiescence.[36,37] The quiescent dormancy model is supported by findings of suppressive matricellular proteins in the pre-metastatic niche coinciding with failure to establish macrometastases.[34,38] As will be discussed, whether the cells are cycling or quiescent during dormancy would affect their ability to be targeted by cycling-dependent agents such as those disrupting DNA replication or intermediary signaling pathways for mitogenesis (such as raf and MEK inhibitors in use for melanomas).

This also raises the questions of what signals keep the cells in quiescence, and which 'awaken' these dormant metastases. It appears that the (re-)expression of E-cadherin coincides with entering dormancy,[8,18] but does not dictate quiescence as cells expressing surface E-cadherin demonstrate mitogenesis particularly notable in the early stages of melanoma- and carcinoma-genesis. Importantly, this emergence from dormancy occurs along with a second cEMT shift.[8,39] Initial studies suggest that stressors of the microenvironment, whether inflammatory cytokines or immune activators, can induce cEMT even in well-differentiated carcinoma cells.[40–43] This is supported by our finding that stressed endothelial cells activated a emergent or mesenchymal phenotypic shift in an E-cadherin-expressing breast carcinoma cell line via secreted growth factor,[40] and work by others implicating a role for endothelium undergoing sprouting as driving tumor emergence.[34] Additional signals can derive from activated stellate cells and macrophages to promote this same shift.[41,42] Interestingly, using an *ex vivo* model for dormancy and emergence, we have reported that outgrowth is reflected in a globally inflamed organ (as denoted by increased levels of cytokines, chemokines and growth factors) while dormancy occurs in the setting of an organ that is 'quieter' than normal homeostasis.[44,45] This evidence that emergence results from, or at least along with, chronic inflammatory activity, along with recruitment of immune cells should have implications for the sensitivity of such emergent growths to immunotherapies.

Investigative models

The study of therapeutic resistance is hindered by model systems that truly reflect either the development of metastases or the treatment of such in humans. Examination of patient specimens is capable of capturing the heterogeneity both between and within patients but suffers from a number of limitations. The mains ones are (a) static sampling and thus not open to determining cancer plasticity, (b) inability to evaluate the earliest stages which are undetectable, (c) interventions limited to singular regimens, and (d) selection bias. To overcome these, a number of models have been developed to isolate specific aspects of metastasis and therapy, each with their own benefits and limitations.

'Spontaneous' rodent metastasis

There are a number of variations of these models. The basic concept involves tumors that develop in mice usually due to genetic engineering to express a specific oncogene in the target tissue; examples include the V600E melanoma[46] and various hormone-driven oncogenes in breast cancer[47,48] or

prostate cancer.[49,50] Chemically induced tumors include those of the liver and other organs. In a few cases, the tumor has developed spontaneously in a particular strain, such as the B16 melanoma, and then is re-introduced into syngeneic animals. For the genetically engineered models, the tumor biology follows from the specific mutations made, and thus only reflects a subset of patients.

These whole animal models benefit from being in intact (i.e. immunocompetent) animals and capturing carcinogenesis and progression. The endogenous nature, or syngeneic aspect when transplanted, allows for a complete immune system response, including acquired immunity, even if the murine immune systems are not directly replicative of the human situation. A major limitation is that these tumors often do not metastasize at high frequency or predictably. This may be overcome by isolating tumor cells from metastatic target organs and re-introducing them orthotopically with serial enrichment yielding more pliant models.

'Inoculated' rodent metastasis

Rodents are used as the hosts of transplanted tumor cells for two reasons in metastasis research. In the first, it is to directly inoculate tumors into ectopic tissues, providing for grafting of tumor cells in organs of metastasis; this is most often published as injection into the tail vein to obtain tumors in lungs, and into the left ventricle to obtain tumor cells disseminated widely with a high predilection to bone. In the second aspect, it is to seed human tumors to study the heterogeneity of human specimens or the *de novo* nature of carcinogenesis unique to humans; these tumor xenografts can be from cell lines or directed human specimens (PDX) either orthotopically or ectopically. Of course, these two aspects are not exclusive, with human tumor cells often being targeted directly to metastatic organs.

The advantage of the direct inoculation into ectopic organs is that the tumor reaches the ectopic tissue at predictable rate and time, enabling tracking of specific stages of metastases. This has been used to study both longer term outgrowth and treatment and even the initial minutes to hours of seeding. In a seminal study, B16 melanoma cells were introduced via the portal vein into the liver to track their fate over the ensuing hours to days to weeks; this demonstrated a high rate of extravasation but $<1\%$ long-term outgrowth rate even for this clonally selected highly aggressive tumor.[28] Such approaches have been extended to metastatic seeding in the brain.[29]

Despite these advantages, this 'directed metastasis' approach opens the question to whether the normal pathological processes are overwhelmed and thus the results are skewed. While the very low efficiency, often on the order of 0.1% of inoculated cells resulting in macrometastases, reflects the likely human situation, the rapid appearance of the macrometastases belies the clinical experience wherein even 'rapid' recurrences require months to years before reaching similar sizes. It is quite likely that the large bolus of extravasating cells overwhelms the pathophysiological responses that drive the epithelial reversion and dormancy noted when limiting numbers of cells are introduced. The quantitative nature of this balance is noted in tumor cells producing greater numbers of EV than the host parenchymal cells, but with this being counterbalanced by the numerically vaster numbers of host cells upon single cell seeding.[35] Similarly, the growth factor-rich milieu of tumor cells may also overwhelm the death signals from the innate immune response to foreign bodies/cells, enabling greater survival of mesenchymal aggressive tumors. This is important in studying resistance to therapy if the surviving and outgrowing 'metastatic' tumor cells have not upregulated key molecules and behaviors that normally function to enable metastatic seeding, and thus would appear as falsely sensitive growths.

Spontaneous metastases from orthotopically placed tumors avoids this 'bolus' effect. The resulting metastases undergo the pathophysiologically relevant phenotypic switches and more likely reflect clinical situation.[51] For syngeneic rodent tumors, this includes the input from the innate and acquired immune systems (and subsequently allows for examining immune-modulating therapies), but still suffers from the limited diversity of rodent tumors and translatable discrepancies to the human immune system. For human xenografts, this captures the clinical heterogeneity and can parse out those cells that have metastatic capacity. However, the immune-deficient hosts that are required obviate the input from any acquired immune system inputs or the assessment of such tumor vaccines or lymphocyte-mediated immunotherapy (the immunologic competence or completeness of 'humanized' mice hosts is still uncertain and usually does not match the transplanted immune system with the particularly tumor cells). Even the innate immune response may be limited in these hosts, though the *nu/nu* mouse system appears more robust and replete than the more facile NOD SCID mice.[52] The involvement of the immune system inputs in metastasis and therapy are open to question as there are subtle but important differences between rodents and humans; for melanoma in particular this is amplified by the overwhelming predominance of gamma-delta T cells in the skin site of melanoma-genesis and invasion, versus the human situation in which the adaptive alpha-beta T cells are the vast majority in these locales.[53]

Ex vivo models of metastasis

Ex vivo organotypic models can provide an all human context as the human microenvironment has species-specific signaling, and many of the newer biologic and immunologic therapies are optimized to work with human sequences. Equally important, this approach can allow for near continuous assessment of the metastatic cascade, with repeated imaging of the same micrometastasis. Sacrificing the entire animal model for a more limited organ tissue allows for such analyses. The details of these different models vary but certain limitations are shared including (a) lack of input (soluble and vesicular signals and hormones) from other tissues, (b) limited immune system presence and functioning, and (c) time-span limitations of days to weeks.

The simplest replete models are tissue slices, most often of brain or liver, onto which tumor cells can be directly seeded. These allow for limiting the number of cells to

reflect the clinical situation and following multiple individual events. As brain and liver are two common organs for metastases and are often the ones of worst prognosis, the clinical relevance of attacking these micrometastases is obvious. Two other commonly involved organs, lung and bone marrow, do not lend themselves to tissue slices due to the physical nature of air/tissue and bone/marrow interfaces, respectively. Compromising the utility of these models is the lack of fluid flow through the tissue that leads to hypoxia and buildup of toxic metabolites that limits the functionality to days.

Recent developments in microphysiological systems (MPS) provide the opportunity to overcome some of these challenges. MPS are complex tissue constructs, generated from individual components (rather than fragments of human tissues), and maintained under engineered conditions that provide for continuous circulation of media, to avoid static conditions that plaque the tissue slices. The cells are either primary human, or more often for ease and reproducibility nontransformed cell lines or differentiated iPSC. The former can be highly limited (except for skin cells), while the latter two groups are not fully mature, functional and differentiated. On a positive note, the media flow can be regulated to deliver nutrients and therapeutic agents in a manner reflecting the human physiology or the pharmacokinetics of drugs. This enables these constructs to function for over a month, a period sufficiently extended to study by drug efficacy and resistance, and secondary challenges.[44,45]

Again here, the most advanced of these MPS are skin, neuropile/brain and liver. While the skin organ cultures have been used to study melanoma invasion,[54,55] the greater potential for examining the behavior of tumor metastasis lie in the target organs. As these MPSs are nascent, there are few studies investigating metastatic behavior, though even these have led to new insights. It was with an early liver MPS that the tight junctions that metastatic carcinomas can form with parenchymal cells were first reported,[56] and that the epithelial reversion was dissected.[18,35,57] The construction of the current MPS and the complete complement of parenchymal and nonparenchymal cells (including immune cells) is described elsewhere.[5,58] These studies in the liver MPS have recently been used to examine the role of inflammation in driving emergence from dormancy,[41,44,45] with findings reflecting those suggested by clinical experience and animal models.[34,38]

Resistance to therapy

The greatest challenge to treating metastases is that these appear to be inherently harder to kill or control than the primary tumors from which they arise. This is not often able to be determined directly, as primary tumors are mostly found prior to clinically evident metastases and removed. However, in clinical situations where tumors are treated along with synchronous metastases or as neo-adjuvant prior to removal, it appears that the disseminated cells are less responsive. A large, early meta-analysis of neo-adjuvant therapy for breast cancer found no increase in overall survival despite strong responsiveness of primary cancers.[1] Even when both the breast primary and positive lymph node nodule respond the adjuvant systemic chemotherapy with complete pathological remission, the cure rate is far less than complete.[3] These insights suggest a survival advantage for the disseminated cancer cells. Herein, we will provide a model, based on preclinical studies of micrometastases to account for this resistance to therapy.

This discussion will focus on generalized resistance to therapies. This is distinct from selective resistance that often appears in the surviving cells after a particular agent is used, and is usually accompanied by a genetic change that may confer survival; this is often selected in preclinical models by repeated exposure to subtotal death challenges. A long-standing well-documented situation is the genetic amplification of DHFR (dihydrofolate reductase) to escape killing by methotrexate; a property exploited in genetic engineering of cells. Other situations include target molecule point mutations to render them impervious to the agent (tubulin mutations to render taxanes ineffective), upregulation of P-glycoprotein/MDR1 drug efflux pumps (for many chemotherapies such as doxorubicin), loss of the molecular target of the agent (one example is HER2, accompanied by upregulation of related oncogenes), and second molecule mutations to compensate (this is the mechanism for vemurafenib resistance by commensural NRAS mutation). While overcoming such specific survival mechanisms are presumed to be critical, the combination approach to most disseminated cancers makes these individual changes less likely. The latter points to either a non-genetic adaptation or a protective effect in the disseminated cells, which is the aspect that will be proposed herein.

It has become accepted that the microenvironment contributes to the generalized chemo- and immuno-resistance of metastases.[59–62] While much work has focused on specific cancer cell intrinsic mechanisms for resistance to individual agents as noted above, the issue confronting our patients is generalized therapeutic failure. This pan-resistance appears to be due to trophic factors and signals from the microenvironment[61,63–67] that drive changes in the cancer cells and the microenvironment itself.[68] What remains unclear is if a particular tumor is protected by specific factors (e.g. TGFβ production or collagen-mediated suppression), or if there is a progression wherein during the early stages of seeding E-cadherin-mediated signaling comes to the fore while larger emerging metastases drive stromal reactivity to provide protective factors such as tenascin C.[15,55,69] As there is little evidence of trophic changes in the earliest single or few cell stage and the carcinoma cell phenotype is plastic,[7] we posit that the microenvironment and the cancer changes in response to this drive different levels of therapy resistance at each stage of progression (Figures 2 to 4).

(Limited) Resistance in the primary site

The primary tumor site is often the most sensitive to therapies (Figure 2), though the usual approach is to remove surgically, particularly for melanoma, or radiologically in some internal carcinomas. However, in some cancers,

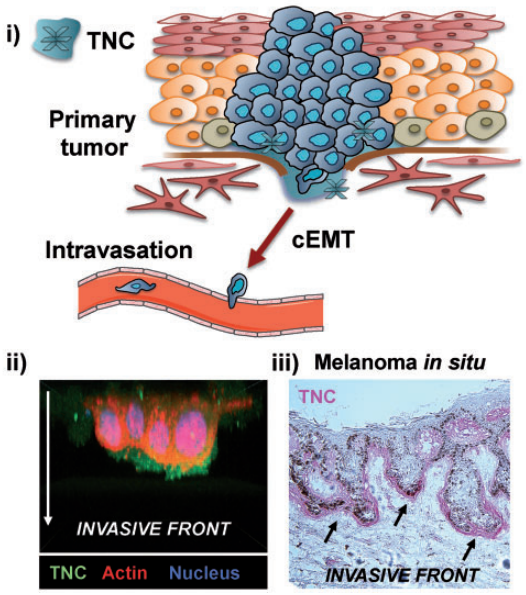

Figure 2. Mechanisms of therapeutic resistance in the primary tumor. (i) Escape from the primary tumor site is promoted by the tumor microenvironment being altered with upregulation of motility-promoting matricellular and matrix-associated proteins (e.g. TNC). TNC is at the invasive front as demonstrated in (ii) an invading melanoma cell lines and (iii) a patient melanoma specimen (adapted from Grahovac et al[55]). However, as these cells are highly active, these protections from death are minimal in the pre-disseminated site.

neoadjuvant therapy is being used prior to surgical removal. These clinical experiences have shown remarkable responses in primary breast cancers shrinking and disappearing[1,3] even though the impact on overall survival is small to non-existent. Furthermore, clinical experience with carcinomas with synchronous metastases (and therefore often not surgically resected) often shows a differential responsiveness, as hinted at by the dichotomy of complete pathological response of primary breast cancers to neoadjuvant therapy with limited impact on clinically undetected metastases that drive the overall survival rates. This is noted with both chemotherapies in which metastases persist through treatment,[71] and even killing by cells of the innate or acquired immune system.[72,73]

Still, a fraction of the invasive tumor cells are capable of surviving lessened cohesion to cells (and the survival signals from those connections) and transit through an ectopic environment/barrier, the dermis for melanoma. While much of the invasiveness appears to be syncytial, bringing the orthotopic support systems, amoeboid motility of the melanomas allows for separation at the front. In breast cancer it has been noted in experimental systems that disseminating cells break from the nodule to migrate and intravasate separately.[74] Both the escape and survival during the process are promoted by the tumor microenvironment being altered with upregulation of matricellular and matrix-associated proteins normally present during wound healing.[54,69] Not only do these proteins promote migration and invasion, but also impart survival advantages.[75,76] However, as these proteins are limited to the leading edge of these invading tumors,[55] the protection is likely limited.

Avoidance of the micrometastases

Successful seeding of the ectopic site, with less than 1% of the cells that reach to target organ, is the most rate-limiting step in the metastatic cascade.[28,29] Thus, the survival mechanisms are either present in a small fraction of the disseminated cells, or only a limiting number of even clonal cancer cells can undergo changes sufficiently rapidly to survive. We posit that this involves the phenotypic switch that is linked to dormancy[7] (Figure 3).

Disseminated tumor cells face a hostile microenvironment in which the orthotopic matrix and cell composition is lacking, and thus the cell is absent the trophic factors that sustain homeostasis in the original tissue. Cells can overcome this in a number of ways. First, they can produce some of the factors themselves, such as secretion of tenascin C by melanomas[15,54] and other cancers. In addition, signals from the metastasizing cancer cells alters the host environment to produce some of these 'wound' matrix components.[35,77] A second mode is to switch to a low metabolic state by entering quiescent dormancy; this requires a reversal of the autocrine growth factor signaling that characterizes the initial cEMT and drives proliferation.[9,21] Thus, the successful micrometastases avoid the starvation of loss of trophic factors.

Another challenge is the nonspecific inflammation triggered locally by intravasation and intercalation of the tumor cell into the ectopic parenchyma. This releases both soluble and cellular challenges. Part of the escape from the cellular attack is for the metastatic cells to be immunologically invisible. There is mounting evidence that PD-L1, which imparts aggressiveness to cancer, is downregulated in successful micrometastases of prostate and breast carcinomas,[78,79] and likely also in melanomas.[80] Interestingly, this immune escape may be linked to the cMErT via limited maturation of PD-L1; EGF receptor signaling drives N-glycosylation needed for cell surface presentation of PD-L1,[81] but during cMErT EGFR signaling is limited.[9]

Worse than immune-escape is the situation of paradoxical response of aggressive outgrowth in response to immunotherapies, a situation seen in up to one quarter of patients and termed hyperprogressive disease.[82] A recent clinical report has described a cohort of 406 patients having advanced non-small cell lung cancer and treated with PD-1/PD-L1 inhibitors in which 13.8% experienced hyperprogressive disease which was correlated with a significant overall worse survival (3.4 vs. 6.2 months).[83] This situation has also been noted anecdotally in melanoma.[84] While the mechanism of this hyperprogressive response to presumed anticancer agents remains unclear, the proposed model suggests a number of testable hypotheses that are concordant with an initial suggestion that activation of the microenvironmental tumor associated macrophages contribute to this perverse outcome.[85] This would represent yet another mechanism for escape from treatments.

Successful seeding also likely requires acquisition of positive signals and not just limiting damage. This is likely provided by the E-cadherin signaling upon heterocellular ligandation after cMErT.[21] E-cadherin ligandation leads to a low-level tonic activation of the canonical

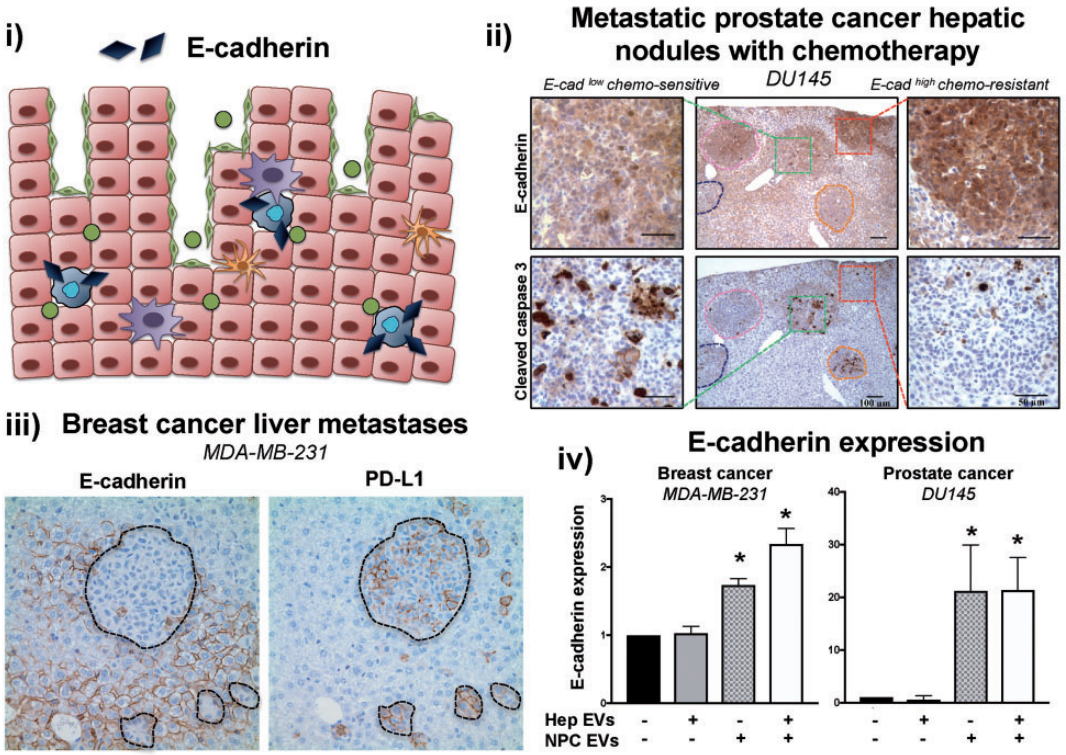

Figure 3. Mechanisms of therapeutic resistance in the micrometastases. (i) Micrometastases escape therapy by upregulation of survival and downregulation of target molecules. (ii) Tumor cells expressing high levels of the E-cadherin in the liver are more resistant to chemotherapy than those expressing low levels (adapted from Ma et al[70]). (iii) An inverse relationship between E-cadherin (chemoresistant) and PD-L1 proteins (here shown in experimental metastasis models in the mouse, unpublished data) would obviate immunotherapy targeting. (iv) EVs from the organ tissue of the metastatic site, particularly the non-parenchymal immune and stromal cells, drive the conversion cMErT in colonizing disseminated cells (adapted from Dioufa et al[35]). (A color version of this figure is available in the online journal.)

survival signaling pathways through MEK-ERK and PI3-kinase-AKT,[86] providing for possible survival signaling. We have reported that such mechanisms provide chemoresistance in breast and prostate carcinoma.[68] This protection functions not only *in vitro* but also *in vivo* wherein inhibitors that are not individually effective in limiting or killing metastases re-sensitize the E-cadherin-positive dormant micrometastases to killing by chemotherapies.[70] Thus, the disruption of either of these survival signaling cascades brings the response of the dormant metastases to be similar to that of the mesenchymal aggressive and chemosensitive primary metastases.

Resistance of the macrometastases

|The resistance of dormant micrometastases would be less problematic if the emergent macrometastases were responsive to therapeutic agents. As the lethal outgrowths present a mesenchymal phenotype, having undergone a second cEMT, often re-expressing PD-L1, these should be responsive to chemo- and immuno-therapy. Clinical experience suggests that such outgrowths may be in part responsive, as often these shrink or even disappear upon exposure to various chemo-, biologically targeted- and immunotherapies (Figure 4). However, these systemic treatments are usually not curative.

The almost inevitable recurrence may come from cryptic dormant micrometastases that have avoided therapeutic effects, or from a subset of emergent mesenchymal cells that are protected from killing. If the former, then one would expect the recurrences to display the same sensitivities as the original metastatic growths, whereas if these recurrences derive from surviving cells, a pan- or acquired-resistance should be noted. The localization of the recurrences would not shed light on either of the mechanisms, as even outgrowing macrometastases may harbor dormant nests, and emergent metastases may disseminate and seed secondary sites without undergoing dormancy.[4,87] Clinically, both situations are seen upon treating the recurrences, but most often the resistance profiles are different than the original metastasis,[88] suggesting that the latter situation of an adaptive or selected population persisting.

The growing metastasis appears as undifferentiated and mesenchymal, with reduced to absent E-cadherin.[8] Such cycling and metabolically active cells should be as susceptible to therapies as the primary lesion; however, this is not the case, and not simply due to exposure-related selection or mutation. The reason for this relative resistance may be that trophic factors in the metastatic niche are providing survival signals. A candidate for this would be the matricellular protein tenascin C which is present throughout the metastatic bed of melanomas (Figure 4) and other carcinomas.[12,55] Again here, the ultra-low-affinity/high-avidity EGF-like repeat interaction with the EGFR on the melanoma cell would provide for survival signals. This should be

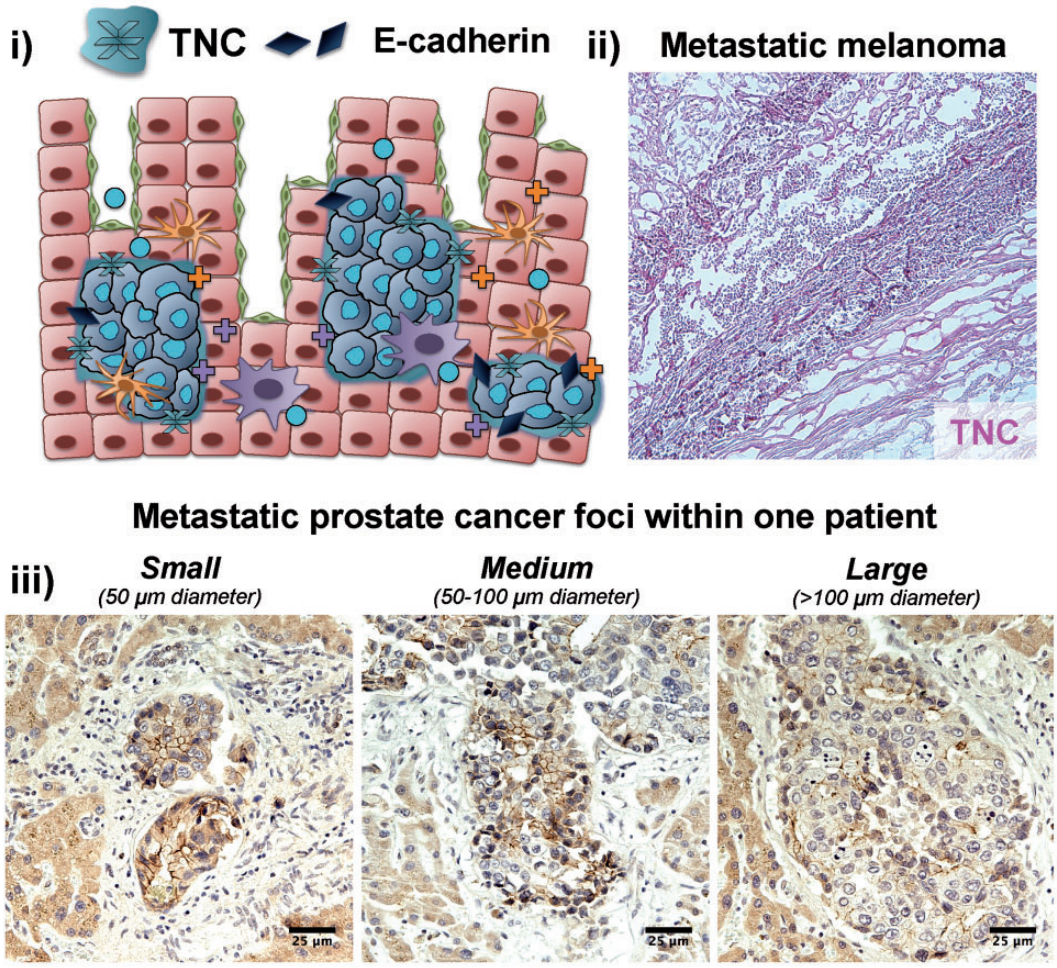

Figure 4. Mechanisms of therapeutic resistance in the macrometastases. (i) Mechanisms of therapeutic resistance persists through a second cEMT and subsequent overt growth. (ii) Therapeutic resistance of tumor cells is maintained via the production of the same matricellular and matrix-associated proteins (e.g. TNC) produced during the first cEMT event for invasion with this now encasing the entire metastasis. (iii) E-cadherin expression inversely correlates with tumor size, with the loss of E-cadherin tracking with emergence as a clinically evident metastatic nodule, opening the way for greater therapeutic sensitivity (adapted from Chao et al[8]). (A color version of this figure is available in the online journal.)

more effective than during initial invasion as the metastases appear to be encased in such a matrix.

Commentary

Metastases challenge our ability to cure or ameliorate the cancer due not only to the geographically widespread and often inaccessible nature, but also because they are inherently more resistant to systemic treatments than the primary growth. This escape from elimination is accomplished mainly through non-mutational events based on the plasticity of the tumor cells. The changes in cellular behaviors and phenotypes, shorthanded as epithelial or mesenchymal based on shape and E-cadherin cell surface presentation or lack thereof, respectively, are imparted in large part by the dynamic microenvironments that the melanoma or carcinoma cells progress through. As part of these switchings, the successful cells are provided with attributes or signals that provide protection from endogenous killing by the innate inflammation of tumor progression or exogenous toxins of therapies. The survival advantage is greatest in the dormant micrometastases that are not only clinically invisible but avoid detection by the immune system, and whose linked quiescence and E-cadherin-triggered survival signals protect against chemo- and targeted-therapies. Protection persists even after active emergence and growth, though to a seemingly lesser extent, again due to changes in the matrix of the metastases, including expression of matricellular protein tenascin C.

This model accounts for the changes in sensitivity to treatments through progression. However, many of these steps have not been demonstrated *in vivo*. The main issue is whether macrometastases recur after treatment due to encompassed dormant nests or from adaptation of a portion of the macrometastases. The basis has immediate implications for developing new approaches to treat these resistant growths. If it is from adaptive changes, then this needs to be accounted, versus the outgrowth of quiescent dormant cells, in which case the challenge is to prevent these cells from being awakened, or resensitizing the dormant cells. If this can be discerned, then we can prevent tumor cells from escaping treatment.

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DECLARATION OF CONFLICTING INTERESTS

All authors declare that they have no competing interests except AW: Patent on LiverChip now being commercialized by CNBio Innovations.

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