4 Heterogeneity and Plasticity of Melanoma: Challenges of Current Therapies

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Abstract: The heterogeneity and plasticity of aggressive melanoma presents formidable challenges in the design of current therapies. Plasticity is defined as the phenotype of cancer cells expressing properties normally related to stem cells, including the expression of genes associated with multiple cellular phenotypes and appearing as undifferentiated, embryonic-like cells. The multipotent phenotype of these tumor cells, expressing vascular, embryonic, and cancer stem cell (CSC) capabilities, offers new insights into their functional adaptation and resistance to current therapies. This chapter highlights major advances in research that (i) help clarify the underlying challenges associated with angiogenesis inhibitor therapy; (ii) discuss important implications of the discovery of reactivation of the normally dormant Nodal embryonic signaling pathway that underlies the CSC phenotype, unregulated tumor growth and metastasis, and resistance to current

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therapies; and (iii) demonstrate the advantage of using combinatorial strategies to effectively target heterogeneous melanoma subpopulations to eliminate relapse and disease progression.

Key words: Chemoresistance; Heterogeneity; Melanoma; Nodal; Plasticity

Introduction

Tumor heterogeneity presents a significant conundrum pertinent to the design of effective therapeutic approaches that mitigate residual disease and progression to metastasis. The complexity of this issue has not been fully appreciated until the dawn of genomic analysis and the revelation of various subpopulations of tumor cells within a tumor lesion expressing multiple phenotype-specific genes and diverse protein markers, especially prevalent in aggressive melanoma (1). At first, these findings seemed enigmatic; however, they prompted further experimental studies into the biological and clinical relevance of a multi-potent or plastic tumor cell phenotype. Most noteworthy, patients with metastatic disease were relapsing following conventional therapies, which strongly suggested the critical need for a refocused approach utilizing targeted therapies.

After years of clinical trials, preventive sunscreen advocacy, and personalized targeted therapies, metastatic melanoma remains the most aggressive and deadly type of skin cancer. In advanced-state metastatic disease, the latest statistics reveal a median overall survival of less than 6 months (2). FDA-approved agents have included a spectrum of products ranging from conventional chemotherapy such as dacarbazine (DTIC) (3), to ipilimumab—a monoclonal antibody that targets the regulatory checkpoint CTLA-4 in T-cells (4), in addition to inhibitors of mutationally activated BRAF (BRAFi) (5, 6), which have been used in combination with trametinib, an inhibitor of the mitogen-activated, extracellular signal–regulated kinase inhibitor (MEK) (7). Additional therapeutic approaches have recently included agents that target the programmed death 1 pathway (8). Despite these noteworthy advances in treatment strategies, an urgent clinical need remains to achieve improved progression-free and overall survival. However, one of the most difficult challenges to address is cellular heterogeneity within aggressive tumors, as depicted in Figure 1. First-line therapies can target portions of a primary tumor, but residual disease can arise from subpopulations of cancer cells with stem cell properties. Metastatic disease can arise from the expansion of cancer stem cells (CSCs) with drug resistance properties, which are not targeted by current therapies. Acquiring a better understanding of the molecular underpinnings of the subpopulations that express a plastic phenotype—and may appear as vascular and embryonic in nature—will lead to the development of new cancer interventions.

Melanoma Vascular Phenotype

The pioneering work of Dr Judah Folkman with respect to tumor angiogenesis initiated a critical paradigm for strategically targeting the blood supply to tumors, and guided the pharmaceutical industry to develop antiangiogenesis agents with

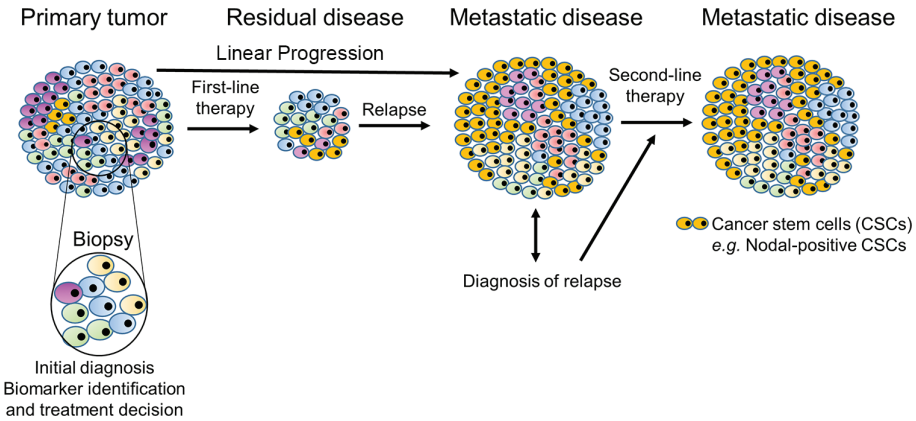

Figure 1 Tumors are comprised of heterogeneous subpopulations of melanoma cells. Generally, a diagnosis from a primary tumor biopsy is based on a "snapshot" of the cellular makeup of a small portion of the tumor mass. Analysis of the cellular composition of the biopsy reveals biomarkers which inform the type of the best front-line therapies suited for treating the tumor. With reduction in the mass of the tumor, cells unaffected by the initial treatment remain and can lead to a relapse of the tumor. Additional biopsies can then lead to second-line treatment regimes. Of note, CSCs, such as those expressing the embryonic morphogen Nodal, that are present in the primary tumor can expand and demonstrate multidrug resistance and lead to linear progression and relapse of the tumor.

the goal of inhibiting growth through nutrient starvation (9). However, as disappointment grew over the outcomes of angiogenesis inhibitor clinical trials, researchers took a closer look at the molecular signature of tumor cells that appeared resistant to this new class of agents. In the case of melanoma, there was confounding molecular evidence, indicating that aggressive melanoma cells express multiple cellular phenotypes, including those closely associated with endothelial cells, epithelial cells, and stem cells—suggesting an unusual plasticity with uncertain significance (1, 10). From a purely scientific perspective, these results were fascinating at the time but fostered serious questions and concerns about cell-type-specific markers that were used to characterize tumor cells versus normal cells. Essentially, depending on the marker selected, melanoma cells could masquerade as endothelial cells because both cell types express endothelial-specific proteins. Most noteworthy, during histopathology examination, tumor cells could be underestimated or at worst go undetected.

When the functional relevance of vascular markers was tested in melanoma models, this resulted in the surprising observation that aggressive melanoma cells expressing endothelial markers can form *de novo*, perfusable, vasculogenic-like networks in three-dimensional culture (3-D), which we named vasculogenic or vascular mimicry (11, 12). Ultrastructural analysis of these networks revealed a remarkable similarity between tumor cell–formed vessels versus endothelial lined vessels, with the exception of the basement membrane lining (13). In tumor cell–formed vessels, blood passes through basement membrane–lined vascular networks with tumor cells sitting exterior to the membrane matrix, while traditional

vessels support blood flowing through endothelial cells lining the vasculature with the basement membrane exterior to the cells. The light microscopic morphological characterization of VM in patient tumors showed matrix-rich channels containing plasma and RBCs lined by melanoma cells, and noteworthy poor clinical outcome in patients where VM was identified (14). Because VM is associated with the aggressive tumor cell phenotype and advanced stage disease, it is hypothesized that this extravascular perfusion pathway serves as a growth advantage and escape route for rapidly growing tumor cells.

When the concept of VM was first presented, it was considered quite controversial (15, 16). However, with the persistent lack of success of angiogenesis inhibitors, the VM paradigm received a serious, second look. Particularly noteworthy was the critical experiment conducted by our laboratory and collaborators, which consisted of a side-by-side comparative analysis of the effects of endostatin (a classical angiogenesis inhibitor) on endothelial cell formation of angiogenic networks versus melanoma cell–formed VM networks (17). In this straightforward experiment using 3-D cultures, the data revealed the inhibitory effect of endostatin on angiogenesis as expected, but melanoma VM was unaffected. This observation prompted further assessment of the underlying molecular mechanisms that might help explain the noteworthy differential response. We chose to specifically measure the integrin α_5-subunit (the endostatin target) expression in human endothelial cells and human metastatic melanoma cells, and found a high level of integrin α_5-subunit expressed (at the gene and protein levels) by endothelial cells and little to no expression of this endostatin target by melanoma tumor cells.

These results provided a substantial explanation for the failure of angiogenesis inhibitors in targeting tumors containing VM pathways, especially prominent in aggressive disease states. Shortly after this revelation, VM was officially adopted as one of the vascular supply routes contributing to the tumor vasculature (18), which would eventually prompt the design of more rational, targeted vascular disrupting agents. This strategic approach was further informed by microgenomics studies conducted by our laboratory consisting of a comparative molecular analysis of laser capture microdissected networks formed during angiogenesis versus melanoma VM (19). These findings revealed factors contributing to tumor plasticity, in addition to documenting important differences and similarities in angiogenesis compared with VM, especially the heterogeneous subpopulations engaged in various aspects of VM. Most noteworthy, new targets for vascular disruption were discovered in this study, which supported the development for a new class of agents.

Melanoma Embryonic Phenotype

The microgenomics study contributed valuable insights into the key players responsible for VM functionality and also introduced a new avenue of investigation in our laboratory focused on understanding the implications of the embryonic phenotype of melanoma, which feature prominently in sustaining plasticity. This direction was also supported by developmental biology findings

showing that cytotrophoblasts engage in VM during the formation of the placenta (20), and accentuated the notion that tumors can recapitulate early developmental events. To gain a broader perspective of the melanoma embryonic phenotype, a molecular comparative analysis was performed on human embryonic stem cells (hESCs) and human melanoma cells expressing the aggressive, multipotent phenotype. These studies revealed the robust expression of a Nodal embryonic signaling pathway in melanoma cells, which was present in the aggressive phenotype but not in the nonaggressive phenotype (21, 22).

Since this was the first description of Nodal in cancer, we searched the literature for information pertaining to its possible function and found the primary resource to be developmental studies (23). Nodal is a powerful embryonic morphogen belonging to the TGF-beta superfamily. It is critical in the maintenance of hESC pluripotency, as well as axis formation and L-R patterning. Nodal can act in an autocrine and paracrine manner, and is largely restricted to embryonic tissues and mostly lost in normal tissues. While hESCs and aggressive, multipotent melanoma cells share Nodal expression in common, only hESCs express the natural inhibitor of Nodal—called Lefty, also a member of the TGF-beta superfamily. Our findings revealed that while Nodal is reactivated in aggressive tumor cells, Lefty is mostly silenced through methylation (24). These observations gave us additional clues relevant to aggressive melanoma cells and the underlying embryonic phenotype. We postulated, and then confirmed, that Nodal expression contributes to the growth of melanoma tumors, and this embryonic signaling pathway is unregulated due to the absence of Lefty, allowing uncontrolled proliferation (22). We also hypothesized that Nodal is a master plasticity gene, based on its quintessential role in hESCs, and we tested this theory by downregulating Nodal expression in melanoma cells and observed a direct impact on phenotype. Specifically, when the melanoma cells no longer expressed Nodal, they acquired a more normal melanocytic phenotype, downregulated their vascular phenotype, were unable to engage in VM, and had a diminished capacity to form tumors (21).

The translational relevance of the Nodal finding was further validated by our laboratory and others using patient tissues and immunohistochemistry (IHC) analyses. Nodal was found to be associated with advanced stages of melanoma, breast, prostate, pancreatic, ovarian and colon cancer, in addition to glioblastoma and neuroblastoma (25). Collectively, these results supported the potential of Nodal as a valuable prognostic biomarker and promising new target to inhibit tumorigenicity and metastasis (26). To pursue this concept, we tested the effects of anti-Nodal antibody therapy on melanoma mouse models injected with metastatic tumor cells. The results showed a reduction in tumor growth at the primary site of orthotopic injection, and a reduction in lung tumor burden in the experimental metastasis model (25, 27, 28). Although these studies were promising, this approach using monotherapy to target only Nodal-expressing melanoma cells did not completely inhibit tumor formation. These data, together with FACS analyses revealing only a minor percentage of melanoma cells actually express Nodal, persuaded us to reevaluate our approach to effectively target aggressive melanoma, which led us to more carefully consider the CSC phenotype in subpopulations of melanoma.

Cancer Stem Cell Phenotype

Guided by the implications of CSCs, as illustrated in Figure 1, expanding their influence during tumor progression because they are able to survive current therapies, we hypothesized that the melanoma cells expressing Nodal would also express a well-characterized CSC marker, CD133, also associated with drug resistance (29). We employed SmartFlare™ technology to selectively sort and study the functional relevance of Nodal subpopulations existing within heterogeneous melanoma cell lines (30). The results indicated that melanoma subpopulations selected for Nodal expression concomitantly expressed CD133 and displayed significant tumorigenic growth in soft agar compared with nonselected cells.

These experiments stimulated a line of inquiry specifically focused on the question of whether current therapies for metastatic melanoma patients were targeting Nodal. Starting with dacarbazine (DTIC), FDA approved in the 1970s, we discovered that the residual tumor cells surviving treatment are strongly Nodal-positive (31). However, a combinatorial approach of treating with DTIC followed by anti-Nodal antibody treatment was most effective in causing cell death, accompanied by the expression of cleaved PARP (an apoptosis marker). Further support for the critical need of new therapeutic approaches, also revealed in this study, showed prominent IHC Nodal localization in patient tissues before and after DTIC treatment. Despite DTIC failing for most patients, it is still used as the front-line therapy in many cases.

Melanoma patients, like many others with cancer, could benefit from targeted therapies as part of the era of personalized medicine. However, despite advances in the field, the heterogeneity of melanoma—especially the CSC subpopulations expressing Nodal and drug resistance markers—complicate our ability to mitigate relapse and progression to metastasis with current therapeutic options. To address this clinical challenge, our laboratory and collaborators examined whether melanoma patients treated with BRAFi therapy experienced a change in the Nodal-expressing tumor cells. The results showed that BRAFi treatment failed to affect Nodal levels in matched melanoma patient samples before and after therapy—that preceded their eventual death due to disease (32). These data encouraged us to perform an experimental assessment using a mouse model with tagged human metastatic melanoma cells, comparing groups treated with monotherapies of BRAFi or anti-Nodal mAb or a combination of both versus controls. The results clearly demonstrated the efficacy of using the combinatorial approach of BRAFi plus anti-Nodal mAb, compared with monotherapy and control. These data provide a promising new strategic approach using front-line therapy together with targeting a CSC-associated molecule—Nodal.

Conclusion

The heterogeneity and plasticity of aggressive melanoma present formidable challenges in the design of current therapies. However, recognizing that cancer cells can reactivate normally dormant embryonic pathways to exacerbate tumorigenicity and metastasis may present a unique therapeutic opportunity. The multipotent

phenotype of aggressive melanoma cells—with vascular, embryonic, and CSC capabilities—offers new insights into their functional adaptation and resistance to current therapies. Considering that aggressive tumors utilize multiple mechanisms to survive and metastasize, it seems prudent to use evidence-based reports to develop combinatorial strategies to effectively target heterogeneous melanoma subpopulations—to eliminate relapse and disease progression.

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