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Therapeutic Implications of Melanoma Heterogeneity

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Abstract

Over the last decade, the treatment of metastatic melanoma has been revolutionized by the translation of molecular insights into therapeutic benefit for patients. These include advances in immune-therapeutic and small molecule approaches aimed at destroying cells with immunogenic antigens or gene mutations. Despite these advances, the limited durability of clinical response and eventual disease progression underscores a need for better understanding of mechanisms underlying tumor development. Current targeted therapies are developed partly based on the rationale that tumors are primarily clonal with respect to mutant oncogene or cell surface antigen target. However, with the advancement of cell isolation and transplantation approaches coupled with deep sequencing and mutation detection techniques, it has become increasingly clear that tumors are polyclonal. As a result, sensitive malignant cells are eradicated by treatment while the remaining tumor cell populations are conferred varying degrees of resistance and survival advantages by harboring or acquiring certain epigenetic and genetic abnormalities. Tumor heterogeneity thus represents a major obstacle to the successful application of current therapies. Gaining insights into the cellular and molecular aspects of tumor diversity will not only facilitate the development and selection of therapeutic targets but also promote the evolution of precision medicine. In this Viewpoint, we will discuss the implications of tumor heterogeneity for the treatment of metastatic melanoma and propose approaches to accelerate the translation of scientific discovery into improved clinical outcomes.

Introduction: Heterogeneity at the Forefront

Melanomas are notorious for being resistant to currently available regimens despite initial response to treatment resulting from death of sensitive cells and manifested by tumor shrinkage. Nonetheless, within a relatively short period of time many melanomas return, often in the form of more advanced disease that is impossible to cure. This effect can be explained by the heterogeneous composition of the tumor containing drug-resistant cells or

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Author contribution

ADB contributed with his expertise on BRAF signaling, melanoma heterogeneity and immunotherapy, including PDX models in writing the manuscript, revised it critically and approved the final version of the manuscript. SJH was responsible for contributing her knowledge on melanoma heterogeneity and BRAF/MAPK signaling and associated therapies, including PDX models in writing the manuscript and drafted the “select clinical trials” table, which ADB finalized and approved. ADB and SJH participated in the revision of the manuscript and supplemental sections, which ADB finalized, approved and submitted the final version of the manuscript.

Conflicts of interest

The authors have declared no conflicting interests.

cells endowed with increased tumor initiating capacity that are missed by therapeutic targeting. Tumors comprise of cell population(s) un-responsive to treatment due to the fact that the transformation process is almost universally multifactorial, yielding malignant tissue composed of genetically and biochemically heterogeneous cells harboring multiple aberrant pathways. Indeed, heterogeneity has long been recognized as a general characteristic of most cancers, particularly melanoma ^{1,2}. Over 100,000 different somatically acquired mutations have been identified in oncology to date and large-scale sequencing studies have revealed extensive genomic heterogeneity in melanoma – with one of the highest rates of mutation of all cancers ³. Moreover, even within genetically homogenous clones, epigenetic regulation results in extensive diversity of cellular populations and provides an additional layer of complexity in the tumor's response to therapy or environmental pressures ⁴. Multiple factors influence the degree of heterogeneity within the tumor, including the tissue of origin, frequency of tumor initiating and differentiated cells as well as extracellular matrix composition ^{5–7}. In addition, gradients of nutrients and oxygen, which are rapidly changing during tumor growth, influence cell cycle activity and trigger either G1 arrest or rapid progression through the S phase resulting in the heterogeneous cell population with respect to its proliferation status. The phenomenon of dynamic heterogeneity, whereby tumors contain areas of actively cycling cells neighboring areas of quiescent cells that can shift depending on selective pressures in the microenvironment, has been extensively studied in melanoma and is often the cause of chemotherapy failure which relies almost exclusively on actively proliferating tumor sub-compartments ^{8,9}. Despite significant progress in our understanding of melanoma genetics and pathogenesis, efforts to develop individualized treatment regimens that lead to durable responses have been hampered considerably by inter- and intra-patient tumor heterogeneity and the complexity of underlying biologic pathways.

Heterogeneity and Targeted MAPK Pathway Therapies: Opportunities and Pitfalls

In the context of therapies targeting genetic mutations in melanoma, those inhibiting the MAPK pathway (which is dysregulated in about 80% of melanomas), have been extensively developed and are represented by small molecule compounds that inhibit the function of the mutant protein or downstream targets in the hope to eradicate the entire tumor ^{10,11}. Vemurafenib and dabrafenib are FDA approved RAF kinase inhibitors that have substantial therapeutic effects in patients harboring BRAF V600-mutant melanoma, with the majority of patients experiencing some degree of tumor regression (see Supp. Table 1 for clinical trial references). Although BRAF inhibitors have striking anti-tumor properties, many patients progress while undergoing therapy typically 6–8 months after the initiation of treatment ¹². Overall, responses are highly variable, complete remission is rare, and 15–20% of patients who harbor BRAF V600-mutant disease do not benefit from targeted therapy at all ¹³.

While molecular mechanisms of resistance continue to unravel and include activation of MAPK-redundant signaling via the receptor tyrosine kinase (RTK)-PI3K-AKT pathway as well as activating mutations in RAS proteins ^{14–19}, new evidence now indicates that in many instances cancer cells lacking BRAF mutations co-exist with their mutant counterparts in the same tumor ^{4,20}. Consequently these cells are completely unaffected by targeted BRAF

therapeutics, putting heterogeneity at the cornerstone of tumor resistance. Compounding intra-tumor heterogeneity, branched tumor evolution via metastasis results in the appearance of inter-tumor lesions that markedly differ in the mutational status of BRAF. Differences in BRAF status between lesions at distinct sites have been reported in up to 44% of melanoma cases, with the incidence of discordance reported in studies increasing as the number of matched samples analyzed per patient increases ²¹. Co-existence of BRAF wild type and BRAF mutant tumor cells within the same patient has important implications for clinical decisions ²⁰. Yet BRAF V600E mutational status is evaluated in the clinical setting using the purely qualitative cobas test on DNA extracted from a single needle biopsy or surgical excision and re-testing for changes in BRAF status at times of disease recurrence or progression is generally not performed. Instead, treatment is initiated based on the findings obtained from an archived tissue sample or a specimen collected early in malignancy that is not representative of the disease in its entirety. These issues underline the need to address intra- and inter-tumor heterogeneity both in the laboratory and at the bedside. A dynamic testing scheme that captures changes in BRAF V600E status throughout disease progression is needed to provide optimal care to patients (See supplemental Text 1 for additional discussion).

Heterogeneity and Immunotherapy: Harnessing Natural Forces

The recognition of melanoma as a highly immunogenic cancer that induces host immune responses, exemplified by frequent regressions associated with lymphocyte tumor-infiltration, has prompted significant research efforts into development of immunotherapies ²² (Select clinical trials summarized in Supplemental Table 1).

Studies aimed at understanding the sensitivity and resistance of melanomas to host immune system led to the discovery that tumor cells often escape immune regulation by expressing antigens that serve to block T-cell activation ²³. Therefore, reversal of this mechanism through application of monoclonal antibodies that target inhibitory immune checkpoints (CTLA-4) or anti-apoptotic pathways (PD-1/PDL1) to increase cytotoxic antitumor response has proven to be one of the most potent immune-therapeutic approaches ^{24–26}. Previously conducted clinical trials on ipilimumab and nivolumab had demonstrated ability of these antibodies to induce objective response (OR) in 10% to 43% of advanced melanoma patients; importantly, combination of both antibodies proved to be most effective resulting in an OR rate of 61% ^{27–29}. While it is preliminary to assess disease-free survival rates, as many of these trials are still ongoing, currently available data from ipilimumab-treated groups indicates that median overall survival (OS) was 9.5 months (95% CI, 9.0 to 10.0 months), with a 3-year survival rate of 21% (95% CI, 20% to 22%) ³⁰. This represents a major breakthrough for otherwise completely incurable disease, but also demonstrates that a significant number of patients are still lost to melanoma, which most likely progresses due to cells comprising heterogeneous tumor populations that were capable to bypass recognition by activated T-cells. Therefore, investigations continue to formulate regimens and cellular therapies that are synergistic with T-cell activating approaches and thus significantly diminish diversity and frequency of tumor clones that could escape the treatment and re-establish the disease.

Intriguingly, new growing evidence suggests that BRAF and MEK targeted therapies can sensitize tumor cells to immune attacks and improve the effector function of immune cells^{31,32}. Analysis of biopsies before and during treatment with BRAF inhibitors has confirmed down-regulation of VEGF and increased tumor-infiltrating lymphocyte density, namely increased CD8+ T-cell density, owing to up-regulated expression of melanocyte differentiation antigens such as MART-1 and induction of class I major histocompatibility complex molecules on BRAF V600E mutant cells³³. MEK inhibitors demonstrate the added bonus of promoting increased melanocyte differentiation antigen expression not just on BRAF mutant cells but also on their wild-type counterparts³¹. In cases where melanoma antigen expression and TILs are significantly decreased at the time of progression on BRAF inhibitors, these biomarkers of improved outcomes are restored when subsequent combined BRAF and MEK inhibitor treatment is initiated. The above observations suggest that both BRAF and MEK inhibitors can increase the overall expression of melanoma tumor antigens to improve antigen-specific T-cell recognition, thus implicating the investigation of combined MAPK pathway targeting with immunotherapy as well as combination BRAF and MEK therapy in the treatment of unresectable melanoma.

Given the significant toxicities associated with conventional immunotherapy, alternative methods to stimulate the immune system to exclusively destroy melanoma are being developed. Immune-therapeutic approaches based on adoptive cell transfer (ACT), which involve engineering a clonal repopulation of anti-cancer T cells for autologous infusion have emerged as a highly promising melanoma treatment based on their unique potential to eradicate cancer cells with relatively mild off-target effects^{22,34}. Notably, response rates are 50% among those patients with melanoma refractory to available therapies irrespective of driver mutation status or level of pretreatment (supplementary Table 1). However, prolonged complete responses in patients have been scarce, necessitating further investigations into tumor pathogenesis and cellular composition in order to understand limiting factors of ACT success. It was recently discovered that melanoma antigens (such as Mart and Tyr) that are often used to prime and selectively amplify reactive CD8+ T cell subsets during adoptive cell transfer are inadvertently missing on the CD271+ tumor initiating/propagating melanoma cells while being present on the more differentiated CD271- cell population³⁵. As a result, patients whose heterogeneous melanomas consist of CD271+ and CD271- cells might experience initial tumor shrinkage after receiving ACT due to the differentiated cell killing, yet melanoma eventually will return stemming from CD271+ cells devoid of antigens recognized by transferred activated T-cells. Even more intriguing was the recent discovery that TNF α , produced in an inflammatory response by transfused engineered CD8+ cells in ACT, induces dedifferentiation and survival of CD271+ melanoma cells with concomitant downregulation of targeted markers, resulting in relapse and eventual disease progression³⁶. Expression of CD271 is highly correlated with ABCB5 that has also been demonstrated to mark a tumor initiating subset in melanomas^{37,38}. Likewise, ABCB5(+) melanoma initiating cells were shown to express significantly lower levels of the melanoma-associated antigens MART-1, ML-IAP, NY-ESO-1, and MAGE-A than their more differentiated progeny. In addition, these cells are capable of dampening activated T-cell response through the inductions of CD4(+)CD25(+)FoxP3(+) regulatory T cells (Tregs)³⁹. These studies unequivocally demonstrate that melanoma heterogeneity driven by the

presence of tumor initiating cells and their progeny, expressing a different repertoire of cell surface antigens, must be taken into account while designing ACT protocols to include activating antigens that cover cell populations fueling melanoma growth and relapse. Particular caution must be observed to avoid destruction of normal tissues/cells while selecting the group of antigens with high avidity to be used for activation of T-cells during ACT, prompting further studies into characterization of cell surface immune-phenotype of tumor initiating cells.

Future Clinical Perspectives: Modeling Heterogeneity in PDX, Où allons-nous?

Coming into the 21st century, we are faced with the fact that only 5% of candidate pre-clinical anti-cancer drugs are eventually approved for clinical use and the chief reason for the high failure rate of new agents is the lack of preclinical models that reliably model to model tumor heterogeneity as seen in human patients. Earlier attempts to create patient derived in-vitro tumor systems relied heavily on clonogenic assays by selectively culturing primary cancer cells on agar in the absence of stromal components^{40–43}. Subsequently, most therapies were developed using established cell lines that were highly responsive to the proposed treatments, whereas the heterogeneous tumor within a patient often represents an entirely different setting^{44,45}. Therefore, the first step toward realizing the goal of developing curative therapies requires adoption of pre-clinical models that accurately recapitulate the marked heterogeneity observed physiologically. Tumors are organ-like structures that exist in intimate relationship with stromal cells and the surrounding microenvironment⁴⁶. Thus, more successful approaches in testing prospective candidate therapies should include a panel of freshly derived patient melanoma cells with respective xenografts in an adapted humanized mouse models in-vivo^{47,48}. Patient-derived xenografts (PDX) from clinical samples have been successfully established during past several decades to study melanoma growth and its therapeutic response^{49–52}. They represent a major advancement toward precision medicine owing to their remarkable ability to maintain the histology as well as genetic and epigenetic landscape largely reminiscent to that of the original patient tumor⁵³. Hence, the PDX models retain patient-like tumor heterogeneity allowing for drug development and efficacy testing toward personalized therapeutic regimens. A recent report using PDXs to study melanoma growth dynamics and its response to BRAF targeting compounds demonstrated that disease progression and appearance of vemurafenib resistant clones seen in xenografts derived from five patients faithfully match clinical observations for those patients while providing insight into resistance mechanisms⁵⁴. A thorough and methodical approach must be taken however every time a PDX is established from each patient. It has recently been demonstrated that extensive fragmentation of the surgically removed human sample during PDX transplantation may result in a number of mouse tumors that fail to capture the full extent of genetic heterogeneity seen in the original patient tumor. Namely, in this study researchers observed an incomplete overlap of BRAF amplification and MEK1T55delins RT mutation between patient sample and resulting PDXs⁵⁵. Therefore, using small tumor fragments from a large heterogeneous tumor to establish PDX can lead to under-representation of genetic anomalies

in the parent tumor and prevent full head to head comparison of PDX to clinical outcome. (See supplemental text 2 for additional considerations on the use of PDX).

In many solid tumors, including melanoma, PDX models were also successfully used to demonstrate existence of tumor initiating cell populations (based on the expression of CD271 and ABCB5) which drive tumor formation through self-renewal and differentiation^{35,38,56–58}. As previously discussed, these cells can activate molecular pathways to avoid recognition by the immune system and are often resistant to small molecule compounds by over-expressing multidrug resistant genes^{59–62}. The failure of initial therapy to completely eradicate these tumor-initiating cells, either within the primary lesion or in a protective metastatic niche, might lead to disease recurrence or progression. Therefore, novel therapeutic strategies tested in PDX and aimed at tumor initiating cells, the source that feeds the tumor mass, administered in parallel with therapies targeting the differentiated bulk of the tumor represent a rational next step toward managing advanced melanoma. Despite continuing debate whether or not frequency of human tumor initiating cells is under- or over- estimated due to the modifications in the mouse xeno-transplantation protocols^{63,64}, their true physiologic frequency could never be determined for cancer patients for obvious reasons. One must realize the fact that the more severely the mouse host is immunocompromised, the higher the frequency of transplanted human cells becomes. Therefore, these models will only reflect the permissiveness of each particular mouse strain, but the true frequency of human tumorigenic cells can never be found due to the fact that human isogenic tumor cell transplantation is not possible. One plausible alternative being rodent strains humanized with fragments of patient skin tissue and/or patient specific hematopoietic system in order to re-establish stromal and immune constituents that influence ability of transplanted cells to initiate and maintain tumor growth^{35,65}. These adapted systems more closely mimic surrounding tumor environment and exert physiologic pressure on the transplanted cells isolated from patient's cancer, allowing close approximation of tumor initiating cell frequency in each particular case. Another vital component in tumor initiation studies is *matrigel* that is widely used during cell transplantation experiments. Profound differences exist between different types of matrigel in terms of concentration and composition of growth factors that can dramatically alter intrinsic properties of transplanted cells. Thus, in the case of increased mitogenic signaling (in the presence of high protein matrigel) and in the absence of immune surveillance in the transplanted hosts these experiments attempt to measure cell's potential rather than physiologic fate when assaying them for tumor initiating properties.

Greater understanding of the cellular and molecular heterogeneity of melanoma is necessary to improve patient outcomes by determining rational combinations of therapies, and developing novel targeted agents. Preclinical testing of these regimens is best achieved in the PDX models that faithfully reproduce patient stromal and immune microenvironment to account for the tissue system-wide response that will ultimately influence tumor growth or regression dynamics. For patient stratification in the clinical setting, quantitative technologies assessing genetic and epigenetic changes in multiple matched samples obtained throughout disease progression are necessary to score degrees of intra- and inter-tumor heterogeneity to unveil significant associations with treatment responses and survival outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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