Intratumor Heterogeneity and Antitumor Immunity Shape One Another Bidirectionally
Yochai Wolf1 and Yardena Samuels2

ABSTRACT

Over the last decade, it has become clear that the genomic landscapes of tumors profoundly impact their immunogenicity and how tumor cells interact with immune cells. Whereas past discoveries mainly focused on the interplay between tumor immunogenicity and tumor mutational burden (TMB), under the assumption that a higher mutation load would give rise to a better patient response to immune checkpoint blockade therapies, we and others have underlined intratumor heterogeneity (ITH) as an important determinant of the magnitude of the antitumor response and the nature of the tumor microenvironment. In this review, we define TMB versus ITH and how the two factors are being inferred from data, examine key findings in the cancer immunogenomics literature deciphering the complex cross-talk between TMB, ITH, and antitumor immunity in human cancers and in vivo models, and discuss the mutual influence of ITH and immunity—how the antitumor response can give rise to tumors with higher ITH, and how higher ITH can put shackles on the antitumor response.

Introduction

The last 20 years have seen a major shift in our understanding of cancer biology, driven by two major advancements: (i) the deciphering of thousands of cancer genomes and their complex landscapes, spearheaded by The Cancer Genome Atlas (1) and (ii) the harnessing of the immune system to counteract cancer by immune checkpoint blockade (ICB) therapy (anti-CTLA4 or PD-1/PD-L1 antibodies) or cell therapy (adoptive transfer, chimeric antigen receptor technology, etc.; refs. 2, 3). As the current ICB therapy given in the clinic has a durable response in only a limited set of patients, with 36% of patients with metastatic melanoma undergoing 5-year progression-free survival at best (4), accompanied by decline in response rates for adoptive cell therapy compared with its success in the pre-ICB era (5), the need for better patient matching using genetic, transcriptomic, epigenetic, metabolic, and proteomic biomarkers that can predict the optimal clinical outcome is needed.

Observations of an initial association between responsiveness to ICB and tumor mutational burden (TMB), especially in tumors with a high TMB—i.e., in melanoma (6, 7), non–small cell lung cancer (NSCLC; ref. 8), and the microsatellite instability–high (MSI-H) form of colorectal cancer (9)—established a common assumption that high TMB confers a better response to ICB therapy. However, this assumption has been challenged by other observations, as we will further discuss herein, which called for the identification of other genetic components and refinement of the concept of TMB in the context of clinical response. One such component is intratumor heterogeneity (ITH), which is now considered a key obstacle to the success of immunotherapy. The interrelations between TMB, ITH, and the tumor microenvironment (TME), with an emphasis on the immune cell compartment, are of prime interest and are the subject of this review.

How Is ITH Defined and What Is Its Evolutionary Role in Tumor Development?

Genetic aberrations, ranging from point mutations to chromosomal rearrangements, gains and losses, are the driving force behind cancer cells’ acquisition of additional genetic alterations and adaptation to new microenvironments (10). Some of these genetic alterations may lead to a fitness advantage that results in the generation of genetically distinct cancer subclones. In the context of this review, ITH refers to the variations in the genomic-driven subclonal structure of a cancer. The degree of ITH reflects the number of distinct clones composing the tumor and the degree of their genetic diversity, the combination of which influences tumor aggressiveness. ITH is further defined by its uneven distribution, spatially or temporally, of its genomic diversification in an individual tumor, fostered by accumulated genetic mutations (11, 12). It should be noted that the very definition of the terms ‘clone’ and ‘subclone’ are somewhat vague, ambiguous, and fluid (13); in principle, since tumors are thought to derive from a single tumor cell, clones and subclones are clusters of cells which have a mutational landscape which has evolved away from the original ‘founder’ single tumor cell.

ITH can be inferred and calculated from genomic data in numerous ways. First, mutation calling can be performed using sequencing data by a variety of bioinformatic tools such as MuTect (14) and be annotated by algorithms such as Oncotator (15). A minimal sequence depth of 100× can be used. Variants supported by 5 reads or more are accepted, and germline variants appearing in public databases (such as dbSNP) are removed. Furthermore, variants identified in sequenced matching normal samples are filtered out as well. A few highly useful computational tools were developed to measure clonal and subclonal mutational composition and evolution and thus
Intratumor Heterogeneity and Antitumor Immunity

I. Introduction

1. Intratumor Heterogeneity

   a. Definition
   b. Importance
   c. Measurement

2. Antitumor Immunity

   a. Definition
   b. Mechanisms
   c. Clinical Relevance

II. The Relationship between Intratumor Heterogeneity and Antitumor Immunity

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   b. Measurement
   c. Clinical Relevance

2. Immunogenicity

   a. Definition
   b. Measurement
   c. Clinical Relevance

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1. Genetic Profiling

   a. Whole Genome Sequencing
   b. Exome Sequencing
   c. Targeted Sequencing

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   a. Tumor-Infiltrating Lymphocytes
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1. Clinical Trials

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   b. Combinations with Other Therapies

2. Personalized Medicine

   a. Treatment Selection
   b. Prognosis Prediction

V. Conclusion

Table 1. Summary of key findings of TMB/ITH interactions with patient survival and ICB response.

<table>
<thead>
<tr>
<th>Ref. no.</th>
<th>Cancer type</th>
<th>ICB type</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>(6)</td>
<td>Cutaneous melanoma</td>
<td>Anti-CTLA4</td>
<td>Association between high TMB and neoantigen load with clinical benefit</td>
</tr>
<tr>
<td>(7)</td>
<td>Cutaneous melanoma</td>
<td>Anti-PD-1</td>
<td>Association between high TMB and clinical benefit</td>
</tr>
<tr>
<td>(8)</td>
<td>NSCLC</td>
<td>Anti-PD-1</td>
<td>Association between high TMB and clinical benefit</td>
</tr>
<tr>
<td>(9)</td>
<td>MSI-H type colorectal cancer</td>
<td>Anti-PD-1</td>
<td>Efficient treatment as first line therapy, unlike MSS type colorectal cancer</td>
</tr>
<tr>
<td>(10)</td>
<td>Colorectal cancer</td>
<td>Anti-CTLA4 + anti-PD-1</td>
<td>Genetic deletion of DNA mismatch repair genes in vivo results in higher neoantigen load and better immune clearance</td>
</tr>
<tr>
<td>(11)</td>
<td>Multiple MSI-H tumors (including MSI-H type colorectal cancer)</td>
<td>Anti-PD-1</td>
<td>~20% of patients of 12 different MSI-H cancers undergo complete response</td>
</tr>
<tr>
<td>(12)</td>
<td>Uveal and cutaneous melanoma</td>
<td>n/a</td>
<td>Uveal melanoma, known to be ICB refractory, is characterized by much lower TMB compared with ICB responsive cutaneous melanoma</td>
</tr>
<tr>
<td>(13)</td>
<td>Meta-analysis of multiple solid tumors</td>
<td>Anti-PD-1</td>
<td>Modest association of high TMB and clinical benefit; better association when TMB is interacting with T-cell-inflamed GEP</td>
</tr>
<tr>
<td>(14)</td>
<td>Colorectal cancer (all types)</td>
<td>n/a</td>
<td>Association with high neoantigen load in both MSI-H and MSS subtypes</td>
</tr>
<tr>
<td>(15)</td>
<td>NSCLC</td>
<td>n/a</td>
<td>High TMB correlated with antigen-experienced CD8 and CD4 TIL phenotype, but also with dysfunctional TILs</td>
</tr>
<tr>
<td>(16)</td>
<td>Cutaneous melanoma</td>
<td>Anti-PD-1</td>
<td>High TMB cannot predict clinical benefit from ICB</td>
</tr>
<tr>
<td>(17)</td>
<td>NSCLC and melanoma</td>
<td>Anti-PD-1 for NSCLC, anti-CTLA4 for melanoma</td>
<td>Tumors with low subclonal neoantigen burden have better clinical benefit, especially if interacting with high TMB; tumors with high subclonal neoantigen burden have poor response</td>
</tr>
<tr>
<td>(18)</td>
<td>cCRCC</td>
<td>Anti-PD-1 and anti-PD-L1</td>
<td>No association between high TMB and clinical benefit, even when TMB is divided to clonal and subclonal TMB</td>
</tr>
<tr>
<td>(19)</td>
<td>Cutaneous melanoma</td>
<td>n/a</td>
<td>No correlation between TIL infiltration and neoantigen count</td>
</tr>
<tr>
<td>(20)</td>
<td>Cutaneous (human and mice models)</td>
<td>Anti-PD-1 and anti-CTLA4</td>
<td>Mouse tumors with increased ITH are less immunogenic and do not respond to anti-PD-1; mouse tumors with low ITH are highly immunogenic regardless of their degree of TMB; high ITH tumors are characterized with lower CD8 T infiltration and higher Treg numbers; human melanoma patient cohorts with low ITH have better overall survival without ICB and better response to ICB</td>
</tr>
<tr>
<td>(21)</td>
<td>Acral, mucosal, and cutaneous melanoma</td>
<td>n/a</td>
<td>No correlation between TIL infiltration and TMB</td>
</tr>
<tr>
<td>(22)</td>
<td>Meta-analysis of multiple solid tumors</td>
<td>n/a</td>
<td>Weak association between high TMB with clinical benefit across cancer types</td>
</tr>
<tr>
<td>(23)</td>
<td>Meta-analysis of multiple solid tumors</td>
<td>n/a</td>
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</tr>
<tr>
<td>(24)</td>
<td>Meta-analysis of multiple solid tumors</td>
<td>n/a</td>
<td>Tumors with low ITH have better overall survival</td>
</tr>
<tr>
<td>(25)</td>
<td>Breast cancer</td>
<td>n/a</td>
<td>Tumors with high ITH are associated with lower survival, lower CD8 and CD4 infiltration, and higher Treg numbers</td>
</tr>
<tr>
<td>(26)</td>
<td>RCC</td>
<td>Anti-PD-1</td>
<td>Low ITH is associated with better clinical benefit</td>
</tr>
<tr>
<td>(27)</td>
<td>Meta-analysis of multiple MSS tumors</td>
<td>Anti-CTLA4, anti-PD-1, anti-PD-L1, and their combinations</td>
<td>Complex association between high clonal TMB and low subclonal TMB and clinical benefit</td>
</tr>
<tr>
<td>(28)</td>
<td>Meta-analysis of multiple solid tumors</td>
<td>Anti-CTLA4, anti-PD-1, anti-PD-L1</td>
<td>High clonal, but not subclonal, TMB predicted better clinical benefit</td>
</tr>
</tbody>
</table>

More importantly, the seemingly intuitive association between the abundance of tumor antigens and a strong immune response is, in fact, disputable, as no correlation between the abundance of antigens, TMB load, or structural chromosomal aberrations and T-cell infiltration or density has been found (52, 53) and no correlation between TMB and neoantigen detection has ever been proven (54). Thus, with multiple reports demonstrating the low predictive power of TMB screening in patients prior to ICB therapy, it has been suggested that the association between TMB and ICB response should be revisited, refined, and reconsidered (55, 56).

At the same time, ITH is becoming a highly acceptable genomic metric that needs to be considered alongside TMB, with significant consequences for antitumor immunity (Fig. 1). A pan-cancer analysis, for example, showed better survival and ICB response in patients with low heterogeneity (25), as was demonstrated also for lung cancer (24), melanoma (21, 57), breast cancer (58, 59), cCRCC (60), and ovarian cancer (61). These observations emphasize the importance of employing ITH and the degree of mutation clonality as an additional genomic biomarker for assessing immunotherapy success.

Interestingly, patients with lung cancer treated with ICB had a better outcome when their neoantigen landscape was enriched with clonal neoantigens and had a worse outcome when it was enriched with subclonal neoantigens (24). A cohort of human melanoma
patients treated with anti–PD-1 also showed better survival with higher clonal TMB (62). These observations were recapitulated in a broader context of a pan-cancer analysis of microsatellite stable (MSS) tumors (63).

In a study correlating genomic factors of seven tumor types (melanoma, head and neck, urothelial, renal, lung, breast, colorectal cancer), it was found that clonal, but not subclonal TMB also predicted positive ICB outcome (64). This finding supports the concept of neoantigen “dilution,” in which strong antigens exert immunogenicity when they are clonally abundant, but when they are subclonal, their ability to induce an effective antitumor response is significantly hampered. Strong evidence for such neoantigen “dilution” was demonstrated in subclonal immunogenic neoantigens detected using HLA peptidomics (65) in SCCs generated from parental melanoma cell lines, which were completely undetected in the parental cell line (21). Moreover, when the clonal fraction of immunogenic subclones in a tumor is “diluted” by mixing them in decreasing numbers with non-immunogenic subclones, the ability to reject the tumor is significantly hampered (33).

Mutual Impact of ITH and Antitumor Response

In ITH research, it is accepted to study the development of ITH in an evolutionary biology perspective (11). Accordingly, ITH and antitumor immunity shape each other in multiple mechanisms of coevolution. Due to selective pressure from the immune system that prunes and eliminates tumor clones with strong neoantigens, the tumor evolves to present novel subclones that, on top of other “traditional” growth advantages, such as excessive proliferative and metastatic capabilities, lower the immunogenicity of the entire tumor, either by the “dilution” of strong clonal neoantigens or by the immune-escape mechanisms of the individual subclones themselves (Fig. 2). This principle is demonstrated in comparisons of the genomic alterations in human melanoma tumors before and after anti–PD-1 treatment, in which ICB nonresponder tumors were found to accumulate novel subclones after treatment, while the responder tumors lost subclones detected before treatment (62). Increased ITH also shapes the immune TME, manifested in the reduced presence and altered phenotypes of immune cells in the tumor. The immune system, on its part, effectively restricts tumor clonality to avoid excessive ITH (66). We believe that understanding the interplay between these concepts is instrumental for the development of future cancer therapies.

A useful readout for tumor immunogenicity is the distinction between immune “hot” and immune “cold” tumors, denoting the high or rare abundance of immune infiltrates, respectively. In a mouse model developed in the Samuels lab that uncouples TMB and heterogeneity by generating highly heterogeneous and highly homogeneous tumor cell lines with varying TMB and injecting them into immunocompetent mice, we showed that tumors with low ITH are more immunogenic and “hot” regardless of their TMB, manifested in increased numbers of CD8+ TILs in situ with better effector function, accompanied by reduced CD4+ FOXP3+ regulatory T cell (Treg) numbers (21). In accordance, in patients with lung adenocarcinoma,
tumor regions with low clonal TMB tend to be “colder” with lower levels of infiltrates, with patients with multiple “cold” regions having a poorer survival rate (67). Beyond the mere presence of TILs in a tumor, their spatial distribution in distinct components of the tumor is also important. In ovarian cancer, tumors with abundant levels of epithelial, rather than stromal, CD8⁺ T cells were characterized with lower ITH and depletion of subclonal, but not clonal, neoantigens, suggesting the immunoediting and selective elimination of subclones with subclonal neoantigens (68).

The association between tumor “coldness” and ITH could stem from the “dilution” of potent antigens. Indeed, it was demonstrated in vivo that such dilution can dampen the antitumor response (33). Alternatively, the association could be due to immune escape, which might be more widespread as the prevalence of minor subclones increases. Clones that escape immune elimination by immune escape, such as immunoediting (the process by which the selective pressure by the immune system reduces the abundance of neoantigens by genomic processes), HLA loss, or impaired antigen presentation, are expected to have a significant evolutionary advantage (3, 68). In accordance, in lung adenocarcinoma, immunoediting continuously takes place in previously “hot” tumor regions that have become “cold,” accompanied by a constant increase in ITH (30). Interestingly, TMB has a nonlinear association with loss of HLA genes, as HLA loss is abundant in tumors with intermediate, but not high, TMB. It is postulated that in these intermediate TMB tumors, other immune-escape mechanisms take place. An intriguing line of research would be to investigate HLA loss in tumors enriched for clonal versus subclonal mutations (69).

When considering the tumor ITH and the immune response, one also must address the heterogeneity within the immune infiltrates, in particular, T-cell clonality. Accordingly, T-cell receptor (TCR) sequencing of CD8⁺ T cells in patients with NSCLC revealed that TCR diversity is positively correlated with nonsynonymous TMB (70). Similarly, the TCR repertoire significantly correlated with clonal composition in ovarian tumors with high epithelial CD8⁺ T-cell infiltration (68). Moreover, minor subclones may alter the TME by secreting chemokines and cytokines that affect the T-cell repertoire within the TME, as was demonstrated in pancreatic cancer SCCs that express high levels of chemokine CXCL1 and block T-cell infiltration and ICB responsiveness in a murine model of pancreatic cancer (71). Minor subclones can also impact tumor-infiltrating immune cells other than T cells. In breast cancer, IL11⁺
clones stimulated IL11R+ mesenchymal stromal cells to recruit neutrophils that can support metastasis outgrowths (35). Interestingly, tumor-infiltrating neutrophils positively correlated with increased ITH in lung cancer (72).

Future Directions—from Basic Research to Clinical Intervention

High ITH is now widely considered an obstacle to cancer immunotherapy (3, 73). Given its importance, it should be factored into patient “tailoring” to immunotherapy. Indeed, it can be harnessed to improve immunotherapy. First, it could serve as a genomic readout alongside TMB, as it was shown that clonal TMB should be segregated from subclonal TMB, as the latter has better predictive power (64). Second, the notion that clonal, but not subclonal, neoantigen burden better correlates with response to ICB highlights the importance of focusing on clonal neoantigens as therapeutic targets (24). Third, understanding how minor subclones can decrease the immunogenicity of the entire tumor in specific cancer types may lead to new therapeutic agents, such as IL11+ subclones in breast cancer, which may be suppressed by future anti-IL11 therapy (35).

Establishing novel mouse models to further distill the role of clonality in tumor aggressiveness as well as deepen our understanding of the relation between ITH and T-cell immunity would be highly beneficial. Such models could be used, for example, to experimentally validate the contribution of tumors with decreased ITH to the mediation of an antitumor immune response, by elevating the clonal fraction of certain clones in the tumor in a controlled fashion. Such models could also be used to control the relative percentage of each SCC in a cell mixture. Namely, they could aid in the investigation of the hypothesis raised in this review that elevation of the relative representation of particular SCCs will expose them to higher cytotoxic activity and neoantigen-mediated elimination by T cells, while lowly represented SCCs will reduce their exposure and, thus, elimination by T cells.

Importantly, these highly controlled mouse models will greatly aid in assessing the effect of the tumor composition on and its response to cancer synthetic peptide vaccines containing neoantigens presented on the tumor cells, in the presence and absence of checkpoint inhibitors. Of particular interest will be a comparison of vaccinations based on neo-peptides that cover a large spectrum of the neoantigen repertoire (all evolutionary tumor branches) versus ones with only a fraction of the neoantigen repertoire (few branches). This has implications for the clinical use of tumor vaccination, as it may be difficult to identify peptide candidates from all subclones of a tumor or from all subclones of potential metastases. Indeed, such data could define strategies for a potent, rationally designed cancer vaccination therapy that accounts for tumor ITH.

To summarize, increased ITH and the emergence of subclonal TMB with reduced clonal TMB give rise to weaker antitumor immunity, reduced efficacy of ICB, and poorer patient survival. Thus, using total TMB as a sole genomic metric to assess and predict a patient’s benefit from ICB is far from an optimal approach, and ITH must be considered as well. Moreover, ITH and antitumor immunity follow each other in an evolutionary arms race. Finally, a better understanding of the interrelations between ITH and antitumor immunity may shed light on new therapeutic opportunities.

Authors’ Disclosures

Y. Wolf reports a patent for Vaccination with cancer neoantigens issued to Yeda Research and Development Co. Ltd. Y. Samuels reports a patent for Vaccination with cancer neoantigens issued to Yeda Research and Development Co. Ltd.

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