

Cancer Neoantigens and Applications for Immunotherapy

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Abstract

Recent advances in immune checkpoint blockade therapy have revolutionized the treatment of cancer. Tumor-specific antigens that are generated by somatic mutation, neoantigens, can influence patient response to immunotherapy and contribute to tumor shrinkage. Recent evidence demonstrating the success of checkpoint blockade immunotherapy in boosting T-cell reactivity against patient-specific neoantigens constitutes a strong rationale for the development of personalized vaccines against these

nonself peptides. With the decreasing cost of next-generation sequencing, peptide manufacturing, and improvement of *in silico* prediction of peptide immunogenicity, it is increasingly important to evaluate the potential use of neoantigens in both diagnosis and treatment. Specifically, these neoantigens could be useful both as predictors of immune checkpoint blockade therapy response and/or incorporated in therapeutic vaccination strategies. *Clin Cancer Res*; 22(4); 807–12. ©2015 AACR.

Introduction

Over the past two decades, the emergence of treatments targeting genomic alterations has led to the concept of "personalized" medicine. One good example is the use of small-molecule inhibitors targeting BRAFV600E mutations, an approach that has been shown to help shrink tumors (1–4). The success of checkpoint blockade immunotherapy in cancer is quickly reshaping both cancer care and our understanding of the cross-talk between a tumor and the host patient's immune system (5–12). CD8⁺ cytotoxic T cells are believed to drive the effect of tumor shrinkage, as they can recognize and target cancer cells that present tumor-specific antigens such as cancer testis antigens or somatic neoantigens (13). The recognition of tumor cells by this population is associated with exhaustion as a consequence of encountering inhibitory receptor ligands expressed by the tumor tissue, such as PD-L1 or PD-L2. Moreover, activation of CD8⁺ cytotoxic T-cells is accompanied by an accumulation of CD4⁺ regulatory T cells (Treg), which suppress the function of effector T cells (14, 15). Antibody-based checkpoint blockade immunotherapy mainly acts by boosting the immune system to target tumor cells through various mechanisms. For example, the major physiologic role of anti-CTLA-4 seems to be through distinct effects on major subsets of CD4⁺ T cells: a modulation of helper T cell (Th) activity to promote effector T cells and downmodulation of Treg immunosuppressive activity.

Tumor neoantigens are the consequences of the genetic alterations accumulated by cancer cells during the tumorigenesis process. They have been recently demonstrated to arise from various processes that alter the open reading frame (ORF) sequences in the genome. Not only missense mutations but also fusion transcripts (16), frameshifts (17), and stop losses can also potentially create altered ORFs (i.e., neoORFs) encoding novel stretches of amino acids that are not present in the normal genome. With the increasing accessibility to next-generation sequencing technologies combined with bioinformatics improvements, the process of neoantigen discovery has accelerated. The use of whole-exome sequencing combined with *in silico* peptide translation has become a promising approach to detect potential patient-specific neoantigens. Moreover, immunogenicity of the discovered neoantigens can be assessed using peptide immunogenicity prediction algorithms and high-throughput assay strategies.

Cancer Neoantigen Prediction

A number of approaches have been used for identification of candidate cancer neoantigens from genomic sequencing data. NetMHC provides MHC: peptide-binding prediction based on artificial neural network (ANN) training methods and position-specific scoring matrices (PSSM). The algorithm is trained on an exhaustive list of affinity measurements described in the Immune Epitope Database and Analysis Resource (IEDB; ref. 18) and the eluted peptide data from the SYFPEITHI database (19). The predictions achieve an average of >75% confirmed MHC binders following prediction (20). NetMHCpan is a related prediction approach but is much more speculative than NetMHC. Complementary approaches include analysis of tumor transcriptomes, proteasomal processing, peptide stability, and peptide transport into the endoplasmic reticulum. Together, this set of tools provides an increasingly refined picture of the patient-specific cancer antigenome (composed of both neoantigens and immunogenic self-antigens) likely to contribute to tumor recognition by the immune system (21).

Neoantigens are now increasingly recognized as immunodeterminants, as there is strong evidence that they participate in early

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tumor recognition and destruction by antigen-specific T cells in the context of immunotherapy treatment (22, 23). In recent studies, two teams independently reported that tumors accumulating a high number of somatic nonsynonymous mutations were more likely to have durable benefit from antibody-based checkpoint blockade immunotherapy (6, 23, 24). This observation is consistent with the hypothesis that recognition of neoantigens, formed as a consequence of somatic mutations, by the host immune cells are important for the activity of such therapy (25). This is of particular interest as data from The Cancer Genome Atlas (TCGA) and others suggest that tumors with high mutational burden are associated with cytotoxic T-cell markers, such as CD8A expression (6, 26).

Tumors harboring potentially deleterious mutations in the mismatch repair pathway (MMR), base excision repair pathway (BER), or nucleotide excision repair pathway (NER) were found to carry a high number of candidate neoantigens and associated with clinical benefit from immune checkpoint inhibitor therapy (6, 26). High mutation burden is present in other types of cancer such as uterine, bladder, head and neck cancers, and stomach cancer and could portend successful responses to checkpoint blockade immunotherapy. Furthermore, the potential of neoantigens in cancer immunization holds promise as a novel therapeutic modality.

Use of Neoantigens to Inform Therapeutic Decisions

Given that immunotherapies only benefit a fraction of patients, ongoing effort to discover biomarkers predictive for response are critical. A number of such markers have been proposed. For example, gene expression profiles of tumors before checkpoint blockade treatment have suggested that those with a relatively high level of expression of immune-related genes are more likely to benefit from anti-CTLA-4 immunotherapy (27). Early reports also suggested that patients undergoing ipilimumab treatment with an absolute lymphocyte count (ALC) gain of >1,000 cells per μL were more likely to gain clinical benefit and improved survival (28). These findings contrast with results reported by Postow and colleagues that ALC was not specifically predictive of overall survival (OS) benefit from ipilimumab (29). Gross lymphocyte count does not seem to be a sufficient predictor of response. Moreover, as these studies mainly measured variables during treatment, they do not provide information that allows prediction of who will benefit from treatment beforehand.

Interestingly, a reduction in the ratio of intratumoral Tregs to effector T cells (CD8^+ T cells mainly) seems to be the rather consistent hallmark of improved immune response and tumor shrinkage from immune checkpoint blockade. A few studies have evaluated the relative expression of PD-1 ligands (PD-L1 or PD-L2) by immunohistochemistry on tumor cells as a predictive biomarker of anti-PD-1/PD-L1 blockade response (30–33). However, the results remain controversial as different methods and evaluation criteria defining positive staining are used, rendering their comparison difficult. Moreover, PD-L1/PD-L2 expression is directly inducible by immune cells, and thus unstable over time, and might simply reflect the level of immune infiltrate or their activity at the tumor site rather than be predictive for response *a priori*. In fact, the majority of these studies reported that a subset of patients with no detectable expression of PD-L1 still have partial or complete response and

that this marker by itself does not optimally select patients for immunotherapy.

Could the genomic characteristics of tumors predict responsiveness to immune checkpoint blockade? In a recent study, Rizvi and colleagues determined that a high mutational burden was correlated with therapeutic efficacy in non-small cell lung cancer patients treated with pembrolizumab, an anti-PD-1 antibody (24). In non-small cell lung cancers, smoking-related carcinogenesis, and a mutational landscape that features a high level of transversions, are strongly associated with both higher mutation rate and immunotherapy response (Fig. 1). Higher mutational burden, in turn, increases the odds of accumulating more immunogenic peptides. Strikingly, the presence of neoantigen-specific T cells in the peripheral blood of some patients further demonstrates that some neoantigens are capable of inducing T-cell reactivity (24). These findings support the hypothesis that neoantigen-specific T-cell responses could participate in controlling or shrinking tumors (22, 23).

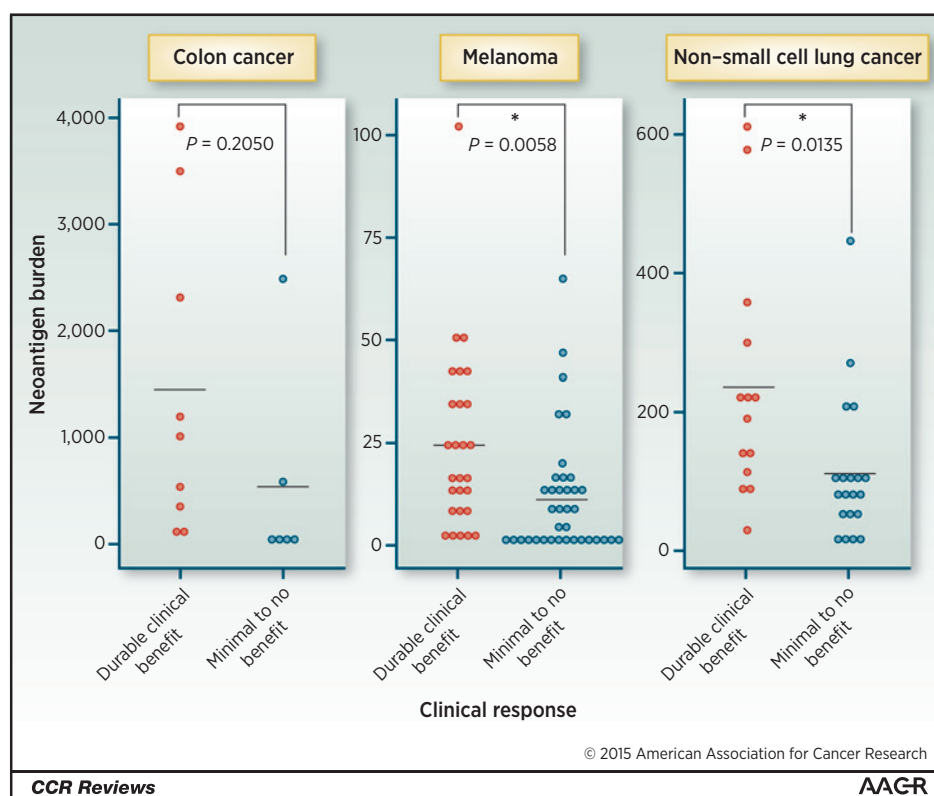
With combination immunotherapy, anti-CTLA-4 plus anti-PD-1, showing an objective response rate and progression-free survival significantly higher than with monotherapy among patients with advanced melanoma (5, 12), the cost of treatment (currently around \$150,000 per course) will probably increase in the near future. Moreover, antibody-based immunotherapy treatments are frequently associated with grade 3 to 4 immune-related adverse events, at a rate of approximately 25% to 50% of the patients in either mono- or combination therapy, respectively. Thus, new biomarkers predictive for immunotherapy response are sorely needed. The accumulated data showing that neoantigen burden has a high predictive value for treatment response highlights their potential to distinguish responders from nonresponders. Combined with the decreasing cost of next-generation sequencing (34), these and other genetic metrics could be incorporated in the treatment decision-making process and increase overall cost-effectiveness of immunotherapies.

Neoantigens and Cancer Vaccine Therapy

Cancer vaccines are designed to boost the immune system's ability to recognize and kill cancer cells. This is done by injecting cancer-specific elements into patients to elicit immune responses against the tumor. In principle, when a vaccine is administered to a patient, components of the vaccine activate professional antigen-presenting cells (APC), including dendritic cells (DC). DCs take up and process the introduced antigens. DCs then migrate to local lymph nodes. Once in the lymph node, the DC displays the antigen on the cell surface through MHC class I or II molecules, presenting them to resting T cells, which become activated. Upon ligation of its TCR to the MHC class I molecule/peptide complex, the neoantigen-specific T-cell is activated, undergoes proliferation, and differentiates into a CD8^+ cytotoxic T cell. It then leaves the lymph node and targets antigens displayed at the tumor surface (35). Similarly, CD4^+ T cells, which recognize peptides in the context of MHC class II molecules can also become activated. Similar to classic vaccines, peptide vaccines can be composed of a number of peptides (36) along with an adjuvant. So far, peptide vaccines have been well tolerated, with few treatment-related adverse events. These approaches are now being adapted to target tumor-specific neoantigens to create personalized therapeutic

Figure 1.

Neoantigen load according to clinical benefit to checkpoint blockade immunotherapy. Data from Le et al., Snyder et al., and Rizvi et al. (6, 23, 24) are shown. Neoantigen prediction algorithms vary in the studies.



cancer vaccines through the formulation and injection of mutated synthetic peptides (Fig. 2).

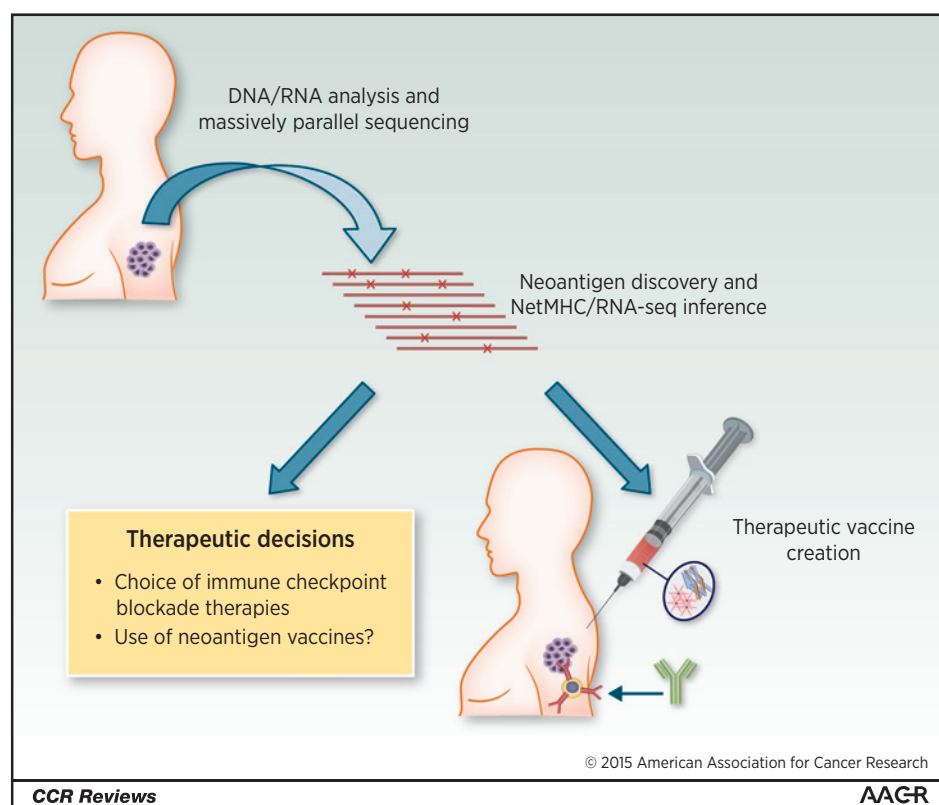
Peptide Vaccination Strategies Targeting Commonly Shared Cancer Antigens

One of the most widely adopted strategies of cancer vaccination is the design of MHC class I restricted peptide epitopes derived from shared tumor-associated antigens, with the aim of activating specific T-cell clones that react against these antigens. Peptide vaccines using shared antigens have been employed as experimental treatments for metastatic melanoma, clear cell renal cell cancer, and other tumor types. They are administered either alone or with an immunologic adjuvant such as a cytokine or Toll-like receptor agonist to boost recognition and uptake by DCs. Monovalent peptide vaccines consist mainly of common antigens highly expressed by melanoma/breast cancer cells (e.g., gp100, MART-1, and HER2 peptides such as GP2 (8, 37–40) as well as cancer–testis antigens normally expressed in immune privileged tissues such as the testis (e.g., NY-ESO-1). Early clinical trials, while potentially promising, gave very heterogeneous results demonstrating substantial immunogenic variability (41, 42). One example is the attempt to use vaccines against chronic myelogenous leukemia (CML), targeting the BCR–ABL fusion oncoprotein. These, either alone or together with IFN treatment, failed to prove a clear clinical benefit (43–46). As a result, none of these therapies are currently in routine clinical use. Explanations for this disparate success could be twofold: First, in some tumors, common antigen expression/presentation may be too low for T cells to initiate an appropriate immune response. Second, it

could be that the tumor is rapidly able to adapt to immunologic selection via an immunoediting mechanism, such as downregulation of MHC or loss of beta-2 microglobulin (B2M) expression, or the establishment of an immunosuppressive microenvironment by both Tregs and myeloid-derived suppressor cells (MDSC; ref. 47). In addition, as the majority of these antigens do not have a predominant or major role in the biology of the tumor, it could be easy for tumors to evade immune surveillance if only limited numbers of epitopes are targeted.

Multiple Peptide Vaccines: The RCC Example

In contrast to vaccine therapy using a single antigen, some investigators focusing on renal cell carcinoma (RCC) worked on identifying multiple, carefully selected shared neoantigens for the purpose of patient immunization. This rationale was based on the hypothesis that vaccine therapy using multiple neoantigens might be more efficient. The hypothesis is that, compared with a single-peptide vaccine, a multiple-peptide vaccine may increase the chance of inducing meaningful T-cell reactivity. Theoretically, this strategy could also lower the chances of tumor clones escaping killing by the immune system. This approach has been investigated in RCC with a selected cohort of 28 HLA-A*02-restricted patients (42). Class I MHC molecules were immunoprecipitated before elution of the bound peptides presented at the tumor surface. This step was followed by mass spectrometry analysis, gene expression profiling, literature-based functional curation, and *in vitro* human T-cell assays. In the end, the investigators identified

**Figure 2.**

A paradigm of neoantigen utility. Proposed strategy to use neoantigen repertoire in a predictive and therapeutic manner. Sequencing of tumor DNA leads to the discovery and assessment of the "neoantigenome." In parallel, creation of a personalized therapeutic vaccination could also be possible.

nine potentially immunogenic tumor-associated peptides (TUMAP), which were shared among the patients' tumors. These peptides were incorporated into a vaccine together with granulocyte-macrophage colony stimulating factor (GM-CSF) as an adjuvant. The authors discovered that patients who responded to multiple TUMAPs were significantly more likely to experience disease control (stable disease or partial response according to the RECIST criteria) than patients who responded to only one TUMAP or had no response. Moreover, in a phase II trial using TUMAP vaccination, together with a single dose of cyclophosphamide in place of GM-CSF, the survival time was extended if a patient had a response to multiple TUMAPs. The authors concluded that the results were promising but that the small numbers of patients evaluated did not enable an accurate assessment of efficacy across RCC. Nevertheless, the advantage of such an approach is that it does not require any *in silico* neopeptide prediction but rather uses presented peptides identified by mass spectrometry. One limitation of this approach is that it requires a relatively large amount of tumor material for proteomic analysis. This approach might not be suitable for some types of cancer or for patients with unresectable disease.

Overall, both mono- and multi-peptide vaccine therapy seems to be well tolerated, with only a few adverse effects associated with the treatment. In the majority of cases, the vaccination process leads to an antigen-specific T-cell response in the peripheral blood of the patient. Thus, immunization using multiple synthetic peptides seems to activate the immune system. The low objective response rates may be due to the presence of active immune checkpoints. Therefore, combined treatment with neoantigen vaccination and immune checkpoint blockade therapy may be an attractive option.

Multiple Neoantigen Therapeutic Vaccination: Where Are We Today?

The results summarized above build a rationale for the further testing of therapeutic neoantigen-based vaccines using multiple targets. The identification of highly immunogenic tumor-specific neoantigens to personalize cancer vaccination appears feasible but much work is needed to define the peptides used and determine how such a therapeutic might be used in conjunction with other therapies (48). Interestingly, neoantigen-based cancer vaccines have been shown to be effective in a proof-of-concept study using mouse models. The authors demonstrated that a therapeutic pipeline based on neoantigen prediction, curation, and peptide vaccination could prevent tumor growth (48, 49). A similar approach has been attempted in humans (Fig. 2), reported by Carreno and colleagues (50). They evaluated the safety, tolerability, and immunologic responses to an HLA-A*02:01-restricted neoantigen-based dendritic cell (mDC) vaccine. They showed that in each patient, T-cell reactivity against predicted neoantigens occurred and that vaccination both stimulated the proliferation of T cells and induced T-cell immunity to several neoantigens. Importantly, the treatments led to the establishment of CD8⁺ neoantigen-specific T cells and memory T cells. Interestingly, they also demonstrated that the TCR repertoire of T cells was broadened upon treatment, suggesting that the treatment elicited neoantigen-specific cytotoxic T-cell reactivity and there was a selection toward the specific clones. Unfortunately, the authors did not measure tumor regression as the patients underwent surgical resection before vaccination. Nevertheless, these results highlight the promise of therapeutic cancer vaccine approaches, and future research will be needed to gauge benefit from this treatment.

Table 1. Examples of some clinical trials involving neoantigen vaccines

Preclinical mouse models					
Author	Neoantigen discovery	Vaccine type	Coadministration of immunotherapy	Efficacy	Reference
Gubin et al., 2014	Whole-exome sequencing + NetMHC ANN	HPLC-purified 8- to 10-mer peptides	Anti-CTLA-4, anti-PD-1	Progressive tumor rejection	48
Kreiter et al., 2015	Whole-exome sequencing + IEDB consensus method	Poly-neo-epitopic coding RNA	No	No significant tumor growth	49
Examples of ongoing clinical trials					
NCT number	Phase	Recruitment	Conditions	Interventions besides neoantigen vaccine therapy	Number of neoantigens
NCT02287428	Phase I	Recruiting	Glioblastoma	Radiotherapy	>10
NCT01970358	Phase I	Recruiting	Melanoma	Poly-ICLC	>10
NCT02129075	Phase II	Recruiting	Melanoma	NY-ESO-1 fusion protein CDX-140, recombinant Flt3 ligand	NA

Abbreviations: HPLC, high-performance liquid chromatography; poly-ICLC, carboxymethylcellulose and polyinosinic-polycytidylic acid.

Several clinical trials using synthetic peptide therapeutic vaccines that include both cancer–testis antigens and neoantigens are currently being tested in patients with untreated solid tumors to evaluate their safety. These trials included patients with melanoma and glioblastoma (Table 1). Eventually, it would be interesting to evaluate the results of this therapy when it is combined with immune checkpoint blockade. If these clinical trials successful, individualized immunotherapy treatment of cancer by combining neoantigen therapeutic vaccination together with checkpoint blockade might significantly improve the current standard of care (48).

Challenges for the Future

How can we improve the utility of neoantigens in cancer immunotherapy? Current studies have primarily used predicted neoantigens resulting from missense mutations (51). One option is to broaden our analysis of potential neoantigens to include other types of potentially immunogenic alterations. For example, fusion transcripts resulting from chromosomal insertions, inversions, and translocations are common in certain cancers, such as CML, lung cancer, bladder cancer, and ovarian cancer (52, 53). Fusion transcripts can encode foreign peptides, and these could be presented on MHC. Therefore, immunogenic targeting of fusion transcripts by vaccine therapy might theoretically be useful. Here, peptide design would be guided by verification of the presence of fusion transcripts from transcriptome data. As no clinical results on the efficacy of multiple fusion neoantigen vaccination are available, these ideas are

simply conjecture at the moment. Regardless, the hypothesis is sound and future studies need to be done to evaluate the efficacy of this approach.

Another potential avenue for improvement of the therapeutic vaccine concept would be the incorporation of MHC class II peptides into vaccination design, which might further improve the efficacy of such treatment. Class II neoantigen prediction and vaccination remain to be explored more deeply in humans. A study by Kreiter and colleagues (49) suggests that a number of the predicted immunogenic neoantigens incorporated into vaccines are recognized by MHC class II molecules on CD4⁺ helper T cells. Unfortunately, MHC class II molecules are highly diverse and more complex than MHC class I restricted peptides. Various algorithms to predict immunogenicity for these peptides are available, but still lack validation. As such, accurate class II prediction remains very difficult. Nevertheless, neoantigen space and its utility in immunotherapy largely remain to be explored. Such work will undoubtedly open new perspectives for the refinement of current cancer vaccine strategies.

Disclosure of Potential Conflicts of Interest

A. Snyder is a consultant/advisory board member for Third Rock Ventures. T.A. Chan is a cofounder of Gritstone Oncology. No potential conflicts of interest were disclosed by the other author.

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