

Recent developments in neoantigen-based cancer vaccines

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Abstract

Cynics point out that a cure for cancer has been “around the corner” for the last 50 years. Nevertheless, the recent convergence of deep DNA, RNA, and proteomic technologies with enhanced understanding of the nuances of the adaptive immune system has generated great optimism amongst researchers. The extraordinary heterogeneity of various cancers, once thought to be a major therapeutic hurdle, may now be bypassed via “personalized” vaccine treatments. Specifically, these treatments involve the identification of MHC-bound peptides that are unique to a patient’s cancer (neoantigens), followed by immunization with peptides, RNA, or DNA that encodes these neoantigens via various delivery systems, thus amplifying the immune system’s response to the particular cancer. Such approaches have shown dramatic results in animal studies. Not surprisingly, then, the field of neoantigen-based immunotherapy has advanced at a spectacular rate, necessitating that interested individuals stay apprised of recent developments. Following an introduction to the subject, we thus focus on aspects that are particularly fast-moving; the cellular sources of neoantigens, which are surprisingly diverse, the tools that are used for their identification, and the status of the numerous clinical trials that are now being conducted.

Key words: cancer, vaccine, immunotherapy, bioinformatics, clinical trials

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List of abbreviations:

aeTSA	aberrantly expressed Tumor Specific Antigen
CTLA-4	Cytotoxic T-Lymphocyte-associated Protein 4
DC	Dendritic Cell
dsRNA	double-stranded RNA
GI	Gastrointestinal
H&N	Head and Neck
hERV	human Endogenous Retrovirus
HLA	Human Leukocyte Antigen
HLA-LOH	HLA Loss of Heterozygosity
IFN	Interferon
MHC	Major Histocompatibility Complex
MS	Mass Spectrometry
ncRNA	Noncoding RNA
NK	Natural Killer (cell)
NMD	Nonsense-Mediated Decay
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Cell Death Protein Ligand 1
RI	Retained Intron

List of abbreviations (Continued):

SCLC	Small Cell Lung Carcinoma
SCM	Splice Site Creating Mutations
SNV	Single Nucleotide Variant
SSC	Splice Site Creation
TAA	Tumor-Associated Antigen
TLR	Toll-like Receptor
TMB	Tumor Mutation Burden
TNBC	Triple Negative Breast Cancer
TSA	Tumor specific Antigen
VEGF	Vascular Endothelial Growth Factor
WES	Whole Exome Sequencing

Introduction

The need for therapies that target cancers with high specificity has long been recognized. Early efforts in targeted therapy focused largely on tumor-associated antigens (TAAs). These proteins clearly served as markers for the presence of various cancers, but non-negligible expression in healthy tissue could not be ruled out. The presence of TAAs in healthy tissue likely explains several disastrous failures in the field.¹⁻³ Even in the absence of dramatic side effects, the disappointing efficacy of some TAA-targeting therapies may now be explained by a stronger understanding of thymic selection, the process by which expression of a large portion of human proteins within the thymus enables self-tolerance to such proteins.^{4,5} The ideal target for therapy, then, would be one that arises via cancer’s well-known predilection for high mutation rates or, at the very least, a target whose cancer-unique signature can be confidently inferred. Such targets are known as neoantigens. In addition to the obvious logic of focusing on neoantigen-based therapies,

a number of studies revealed that the specific T cells responsible for some clinical responses recognized neoantigens.⁶ In parallel, other studies noted that the efficiency of checkpoint inhibitors, drugs that counter a number of immune-evasion tactics seen in cancers, often correlated with cancer “mutation load”; once the immune blockade has been lifted, more neoantigens mean more candidates for T cell recognition.^{7,8} Obviously, identification of a patient’s very particular “mutanome” has only been feasible in the last decade or so, with the advent of cheap, accurate, and rapid exome sequencing.

Neoantigen-therapy is notable for the extent to which a patient’s immune system is coaxed to perform the dirty work of cancer-killing. The essential steps of this process, applicable to any epitopes that are recognized as “foreign”, are illustrated in **Figure 1**. We first note the heterogeneity of cancer. This heterogeneity may refer to DNA mutations or RNA and protein expression differences between cancer types (e.g. breast cancer vs. leukemia), or it may refer to intra-tumor differences.⁹ In any case, a near-unique repertoire of neoantigens is associated with a patient’s cancerous tissue. Such antigens may be released into the blood upon cell death, and/or displayed on the

cancer cell surface in accordance with the peptide-binding properties of the patient’s set of HLA molecules. In the former case, antigen processing and display by dendritic cells serves to activate CD8 and CD4 T cells that survived thymic selection. T cell trafficking, a process often negatively regulated by cancers,¹⁰ then directs the T cells to the cancerous tissue, where interactions with HLA-displayed neoantigens initiate the process of cancer cell killing.¹¹ Cancer-related antigens are then released into the blood, completing the “cancer immune cycle”. Such positive feedback suggests that a simple “jump start”, provided with or without the assistance of various therapies, may be sufficient for an effective cancer response. Inhibition of the cycle is, of course, a tactic employed by cancers. However, a healthy immune system also employs checkpoints to minimize the possibility of autoimmune responses.

The Nuts and Bolts of Neoantigen Identification

An early, two-step schema for the identification of neoantigens is seen in work from the lab of Catherine Wu.¹² The only essential “omics-level” wet-lab protocol was the whole-exome sequencing of both cancerous and germ-line tissue. Sequence

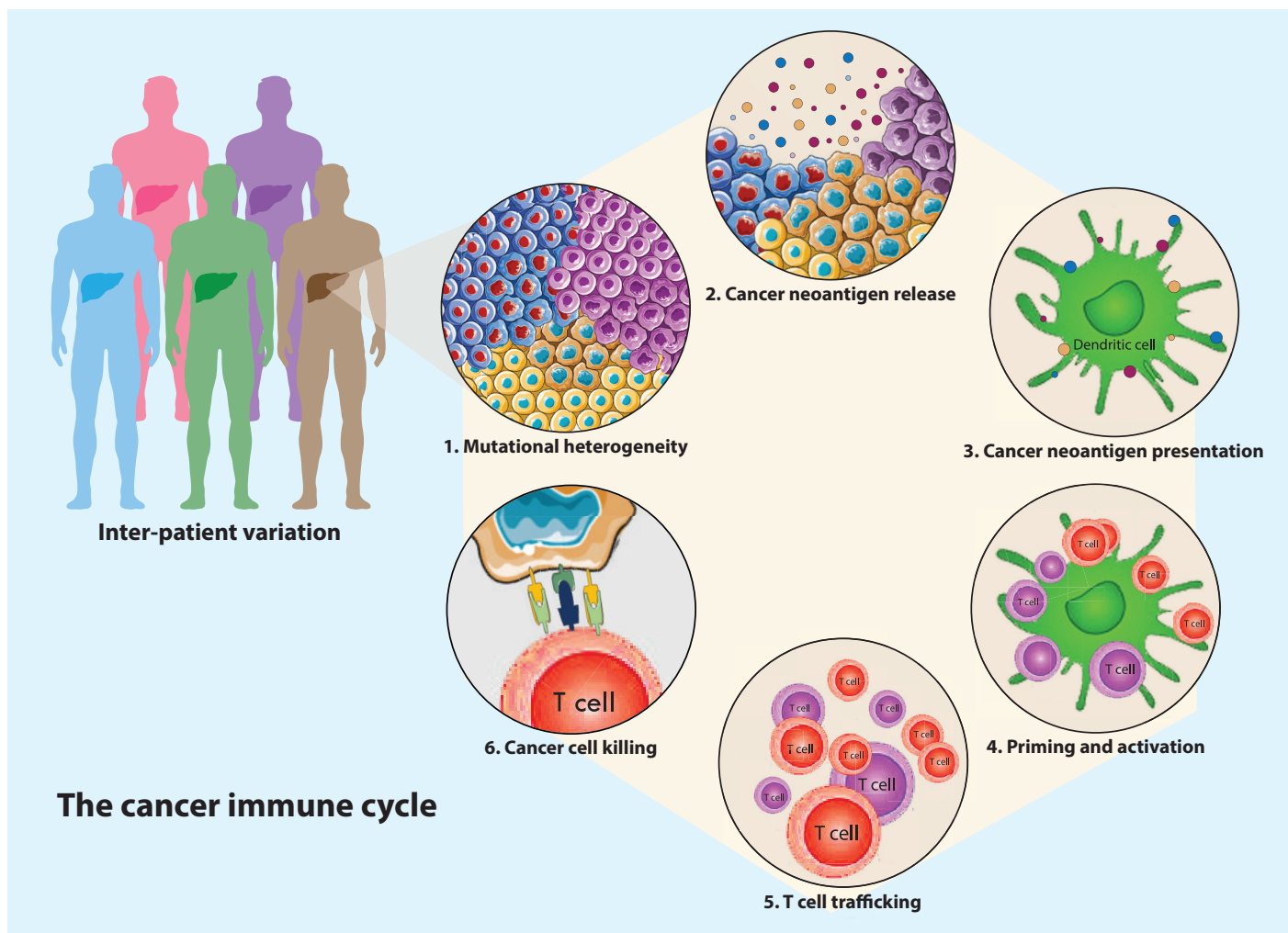


Figure 1. The cancer immune cycle. Throughout the image, different colors represent variation at different levels of the cancer immune response. Patients display great variation in their cancer mutanomes, even between shared cancer types (liver cancer is depicted here). Within a tumor, different colors show mutanome variability, with different tumor clones releasing neoantigens. At the level of the immune response, vast variation (for simplicity, only two different T cell clones are shown) in the T cell repertoire allows for recognition of neoantigens, followed by cancer cell targeting and killing.

comparison allowed identification of cancer-specific non-synonymous mutations via the variant-calling tool, “Mutect.”¹³ Next, neoantigen candidates were computationally screened for HLA-binding affinity. RNA-sequencing was performed only to validate the expression of particular neoantigen candidates. ELISpot assays were performed, with IFN- γ release signaling that a particular neoantigen elicited a T cell response.

Even with the most minimal of protocols, confounding factors arise. In particular, we note the necessity of calling a patient’s specific HLA repertoire amongst the thousands of variants in the human population in order to predict the efficacy of particular neoantigens. Also, given cancer heterogeneity, the likelihood of healthy tissue contaminating cancer samples, and possible inaccuracies in sequencing data, defining and refining the cutoffs at which a mutant may be confidently identified is not a trivial exercise. Currently, a number of tools are available to simplify the process of HLA-typing (see the following reference for a comparison of these tools)¹⁴ and to assess tumor heterogeneity and contamination.¹⁵

Deep RNA sequencing is now routine in most neoantigen-identification pipelines. Given the potential volume of neoantigen candidates identified in the process of variant calling, it is convenient to validate RNA expression prior to HLA-binding assessment. Also, as will be seen below, researchers may wish to examine the possibility of neoantigens being generated

from genomic regions not covered in standard exome sequencing.

Mass spectrometry (MS) is also often utilized in neoantigen identification. Given that neoantigens are peptides, not nucleotide sequences, MS usage is of obvious logic. One might even ask why exome- and RNA-seq are needed in a universe where peptides in cancerous tissue can be compared with peptides in healthy tissue. A number of issues impair the utility of MS, however. Most importantly, MS currently lacks the sensitivity to detect low-abundance peptides. Also, the copious quantities of HLA-specific antibodies required for peptide isolation are problematic. Finally, the apparent absence of a peptide in healthy tissue does not guarantee a system-wide absence. One technology finding increased prominence in neoantigen identification protocols is that of ribosome profiling, which enriches for RNAs that are under translation, revealing the likely translation frame. In some sense, then, “RiboSeq” bridges the gap between RNA-seq and MS.

Figure 2 illustrates a current, basic path toward neoantigen vaccine application. In addition to the bioinformatics-intensive steps discussed above, it is seen that decisions regarding neoantigen delivery remain. Also, appropriate adjuvants that can generate satisfying anti-tumor T cell responses must be chosen. Current trends in both areas are noted below in the clinical trials sections.

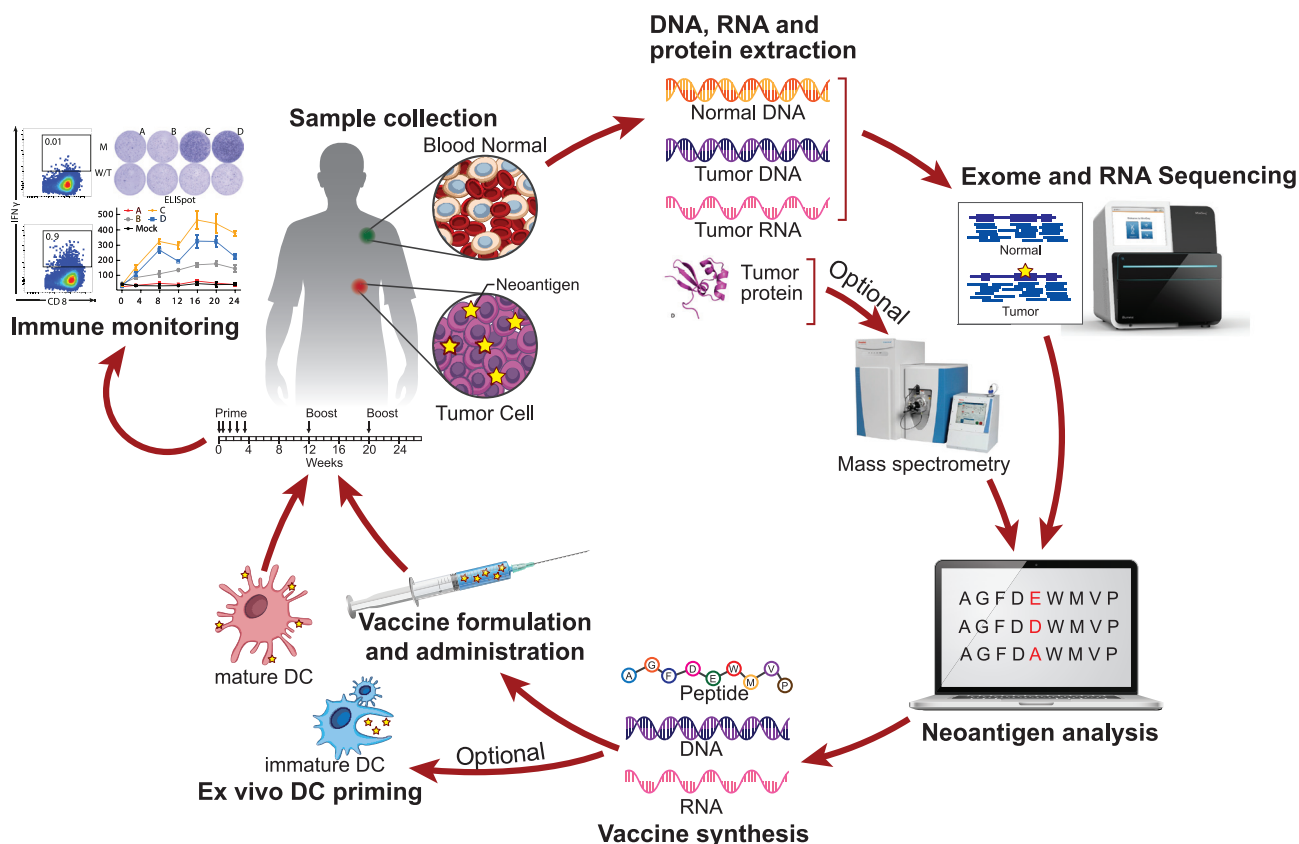


Figure 2. A simple, standard workflow from patient sampling through treatment. Patient cancer DNA is compared with DNA from healthy tissue. RNA sequencing assures that neoantigens of interest are expressed. Mass spectrometry may confirm that neoantigens are bound to HLA. Vaccines may be formulated as peptides, or as the RNA or DNA that encodes the peptide. As with vaccines against infectious agents, adjuvants are typically included in the formulation. Peptides may also be incubated with isolated dendritic cells in order to activate these cells ex vivo before vaccination with these dendritic cells. Note that a checkpoint inhibitor drug is often administered to patients to help alleviate immunosuppression in the tumor microenvironment.

Sources of Neoantigens

Protocols for neoantigen identification have, over their short history, focused on cancer-specific mutations within protein-coding regions of DNA, namely non-synonymous single-nucleotide variants (SNVs) and indels.¹⁶⁻¹⁸ This approach is both logical and convenient, as therapeutic effects depend on cancer-specific display of peptides, and exome-sequencing is now routinely performed without great expense. Nevertheless, numerous researchers have focused on the identification of non-canonical sources of neoantigens. Such a search is necessitated by several factors. Most importantly, following computational winnowing of poorly-displayed and poorly-immunogenic canonical neoantigens, some patients are left without a single vaccine candidate. In one summary of 13 studies, 53 of 1,874 tested SNV neoantigens were shown to elicit a T cell response; on average, fewer than 2 neoantigens per patient were immunogenic.¹⁹ Thus, additional sources of neoantigens

may be necessary in a large number of cases. Also, it is expected that some classes of neoantigens should provoke a stronger immune response than others. For example, a standard SNV-derived neoantigen is characterized by a single amino acid alteration which, given the nature of HLA display and the genetic code, may not differ dramatically from its wild-type relative. A more exotic class of neoantigens, on the other hand, may lack similarity to any peptide found within the healthy proteome, thus possibly provoking a heightened immune response. Finally, though the vast majority of canonical neoantigens are “personal”, i.e. found only on a patient-by-patient basis, other classes may be shared amongst patients, raising the possibility of “off-the-shelf” treatments.

Potential sources of neoantigens are listed in **Figure 3**. Following canonical neoantigen sources, we list fusions, the cancer-specific linkage of regions normally found on separate chromosomes. Such events should frequently give rise to


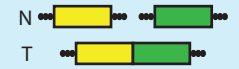
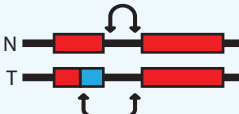
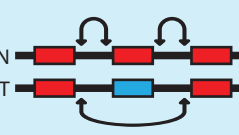
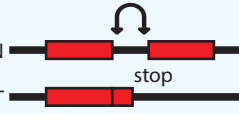
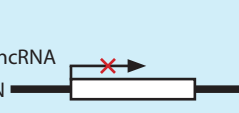
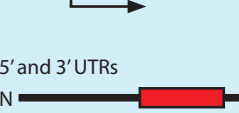
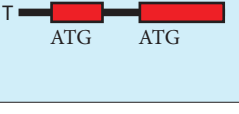
Source	Graphic	Literature Example	Contribution
NON-SYNONYMOUS MUTATED CANONICAL OPEN-READING FRAME: SNVs AND INDELS		<i>Systematic Identification of Personal Tumor-Specific Neoantigens in Chronic Lymphocytic Leukemia</i> ¹²	Wu highlights multiple comparative metrics between cancers. To offer one: melanoma ranks highest in neoantigen counts, with as many as 5,811 in a sample
FUSIONS		<i>The Landscape of Tumor Fusion Neoantigens: A Pan-Cancer Analysis</i> ²⁰	0-25 fusions per melanoma sample
SPLICE SITE CREATION		<i>Systematic Analysis of Splice-Site-Creating Mutations in Cancer</i> ²³	8,656 TCGA tumors examined; 1,964 new splice sites; 10/11 sites validated; 1 peptide identified via MS
ALTERNATIVE SPLICING		<i>Comprehensive Analysis of Alternative Splicing across Tumors from 8,705 Patients</i> ²⁴	an average of 1.7 ASNs (alternative splicing-derived putative neopeptides) in combined BRCA and OV analysis vs. 0.6 SNV-related neopeptides; 15 ASNs observed over multiple samples
INTRON RETENTION		<i>Intron Retention is a Source of Neopeptides in Cancer</i> ²⁵	intron-retention neoantigen candidates are approximately 70% as numerous as standard somatic mutant neoantigens; RI load tends to anti-correlate with response to checkpoint inhibitors
LONG NON-CODING RNA, 5' AND 3' UTRs, HERVs		<i>Noncoding Regions are the Main Source of Targetable Tumor-Specific Antigens</i> ²⁷	40 TSAs (tumor-specific antigens) identified from 2 cell lines and 7 primary tumors, 90% of which were derived from non-coding RNA
		<i>Integrated Proteogenomic Deep Sequencing and Analytics Accurately Identify Non-Canonical Peptides in Tumor Immunopeptidomes</i> ²⁸	approximately 80 tumor-specific aeTSAs identified in one branch of the study; in another branch, 1/500 showed clear immunogenicity via CD8 T cell ELISpot assay
		<i>Endogenous Retroviral Signatures Predict Immunotherapy Response in Clear Cell Renal Cell Carcinoma</i> ²⁹	3,173 hERVs expressed in at least one cancer type; the underlying data does not allow comparison of hERV-derived neoantigens versus other types

Figure 3. Potential sources of neoantigens, prime examples of literature describing their discovery, and some data illustrating the possible contribution of these neo-antigens at the per-patient level. Full titles are given in the “literature example” column to give readers a sense of the subject. For graphics, dotted lines indicate a continuing sequence. PTM; post-translational modification; N; normal tissue, T; tumor tissue, P; phosphorylation. A typical RNA-editing event is depicted, with adenosine altered to inosine. In the final two rows, readers should be aware that neoantigens are generated at the post-translational level, i.e. representations of peptides are shown.


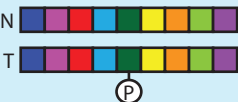
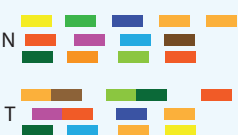
Source	Graphic	Literature Example	Contribution
RNA EDITING AND MODIFICATION		<i>A-to-I RNA Editing Contributes to Proteomic Diversity in Cancer</i> ³¹	no evidence of cancer-specific expression
CANCER-SPECIFIC PTMS		<i>Mass Spectrometry Based Immunopeptidomics Leads to Robust Predictions of Phosphorylated HLA Class I Ligands</i> ³³	2,190 phosphorylated HLA-I ligands identified without phospho-enrichment from 61 samples
PROTEASOME SPLICING		<i>Mapping the MHC Class I Spliced Immunopeptidome of Cancer Cells</i> ³⁶	no evidence of cancer-specific expression
		<i>Estimating the Contribution of Proteasomal Spliced Peptides to the HLA-I Ligandome</i> ³⁷	
		<i>Global Identification of Post-Translationally spliced Peptides with Neo-Fusion</i> ³⁸	

Figure 3. (Continued)

neoantigens at junctions, and potentially downstream of junctions in cases where a frame-shift results. In one study,²⁰ these neoantigens showed relatively high immunogenicity, though shared neoantigens and driver neoantigens tended to show low immunogenic potential. As many as 55 fusions were identified in a single cancer sample. At least two studies have demonstrated immunogenicity for specific fusion-derived neoantigens.^{21,22} Vaccine candidates based on fusions pass the “common sense” test, but are limited by a relative paucity of fusion events versus SNVs and indels in cancer.

The next two sources of neoantigens that we examine both relate to splicing. In the case of splice site creation (SSC), cancer-specific mutations create new unique splice sites and thus neoantigens. In the case of cancer-related alternative splicing, mutations near existing splice sites or within splicing proteins increase the extent to which exons are retained or skipped. The two cases differ in the extent to which one can confidently assume that a potential neoantigen is truly unique to a cancer. In the case of SSC, entirely new exons are found within the final spliced RNA. In the case of cancer-related alternative splicing, however, it is essentially impossible to conclude that a particular alternate splicing arrangement is unique to cancer tissue. Splice-site-creating mutations (SCMs) were investigated by Jayasinghe et al.²³ Bearing in mind that a single SCM can be responsible for multiple neoantigens, 23% of 8,656 tumor samples expressed SCMs. A number of SCMs were recurrent. One particular recurrent SCM, found within the GATA3 gene, was singled out and validated via MS-based discovery. Regarding the potential of alternatively-spliced TSAs, there are reasons for optimism despite the aforementioned caveat. In the premier work on the subject,²⁴ Kahles et al scoured TCGA data and found nearly 3 predicted HLA-binding “neojunction” peptides for every such SNV-derived peptide. Furthermore, a surprisingly high number of neojunctions were recurrent; of nearly 9,000 samples, 7% of neojunctions were seen in more than 100 samples.

Though associated with splicing, we list “intron retention” (RI) as a separate source of neoantigens. Here, failure of splicing machinery or splice-site mutations causes introns to be included in mature RNA, generating neoantigens. In the case of mutation-associated RI, the confidence that the candidate neoantigen is unique to the cancer would be heightened. A study

focusing on intron retention in melanoma tissue was able to increase neoantigen load by 70% via the inclusion of RI²⁵ versus canonical somatic mutation neoantigens. Naively, one might expect that nonsense-mediated decay (NMD) would minimize the presence of RI neoantigens. In fact, one broad screen of TCGA data suggests a general trend toward enhancement, as opposed to dysregulation, of NMD in cancer.²⁶ Nevertheless, a number of these candidates could indeed be identified via MS, though perhaps oddly, a slight anti-correlation was noted between RI load and checkpoint inhibitor benefit.

In examining the potential for neoantigen expression via non-coding RNAs (ncRNA), Laumont et al tackled the differential expression problem by using thymic RNA as the control tissue.²⁷ The premise here is that the thymus expresses a large percentage of known genes given its role in inducing tolerance to HLA-displayed peptides. If identical reads were seen in both cancer and thymic tissue, the reads were excluded. Over a limited number of human cancer samples (four B-ALLs and three lung cancers), 20 of 22 MS-validated tumor specific antigens (TSAs) were not mutation-associated. Rather, these were non-mutated peptides derived from non-coding regions that were aberrantly expressed in cancer (aeTSAs). Similar percentages were derived from the mouse branch of the study, where immunization with TSA-pulsed dendritic cells showed dramatic increases in mouse survival in several cases. It is thus clear that therapy using aeTSAs may provoke life-saving immune responses. Additionally, given that aeTSAs are not the result of mutation, they are expected to be recurrent over multiple patients; recurrent aeTSAs were indeed identified in Laumont’s work. Bassani-Sternberg et al examined the possibility of non-coding RNA as a source of aeTSAs using a different approach.²⁸ Here, ribosomal profiling was used to assay RNAs with which ribosomes interact. Such a method allows the prediction of reading frames, dispensing with the need for in-silico three-frame translation of all RNAs found to be dominant in tumor tissue. It also allows 5’ and 3’ UTRs to be examined for non-canonical translation. Stringent MS analysis of tumor samples versus controls followed, culminating in the identification of about 80 aeTSAs that could not be identified in healthy tissue. A comparison of canonical versus non-canonical TSA yield was only made for several samples, yielding an approximate 1:1 ratio of both. However, of greater than

500 aeTSAs screened, only one displayed strong immunogenicity, with authors explaining that such results may reflect difficulties inherent in current screening protocols. Again, the possibility of shared antigens was emphasized. A final recent work examines a subset of ncRNA, that of endogenous retroviral elements (hERVs), again using ribosomal profiling to home in on regions that may be translated.²⁹ To no surprise, peptides generated from hERVs indeed showed evidence of translation, with tetramer assays showing that peptides derived from a specific hERV, hERV-4700, were recognized by a large subset of infiltrating T-cells. A new tool for the identification of hERVs from RNA-seq data, hervQuant, is provided in the study.

We conclude our examination of TSA sources with three rather exotic sources of potential antigens. In all three cases, cancer immunogenicity would depend on broad biological mechanisms being specifically altered in cancer tissue. For example, RNA-editing is a process by which the sequence of RNA may be altered after transcription. This editing is performed by a limited array of enzymes, most prominent of which is ADAR.³⁰ Though RNA-editing alterations have indeed been recognized in cancer,³¹ a clear “cancer only” peptide candidate has yet to be associated with RNA-editing. Similarly, it is possible that a peptide sequence may be displayed in both normal and cancer tissue, but only the cancer peptide receives, or lacks, a particular post-translational modification. It is clear that modified peptides may be displayed by HLA molecules,^{32,33} but few reports suggesting cancer-specific immunogenicity exist. In one case, glycosylated peptides associated with leukemia could stimulate ELISpot IFN- γ signals in PBMCs from healthy donors.³⁴ Finally, proteasomal splicing is a process by which two independent peptides may actually be ligated after proteasomal cleavage of their original proteins. It is clear that

such peptides may be displayed by HLA.³⁵⁻³⁸ Once again, however, the evidence for neoantigens derived from the splicing of peptides is lacking.

We’ve refrained from listing one final intriguing, potential class of neoantigens in our table, as their utility in vaccines remains questionable: fragmented proteins that are unique to apoptotic cancer cells.³⁹ These caspase-cleavage products have been described as immunogenic, but it remains to be seen if vaccination against a class of peptides generated from cells that are essentially dissolving could be effective. Certainly, “bystander” effects, whereby released antigens are picked up for display on neighboring cells, have been described.⁴⁰

Pre-Clinical Studies

Published pre-clinical studies have demonstrated significant T cell responses after neoantigen-based vaccinations in mice. Yadav et al. injected C57BL/6 mice with MC-38 colon cancer cells and examined the response to neoantigen vaccination in both prophylactic and therapeutic settings.⁴¹ Neoepitopes were derived via MC38 exome sequencing, MHC-I binding and solvent-exposure analysis of candidate neoantigens, followed by mass spectrometric verification. In the prophylactic model, tumor volumes significantly decreased. In two cases where tumor growth was not inhibited, dextramer staining revealed low levels of neoantigen-specific CD8 T cells, suggesting that CD8 T cells were responsible for protection against MC-38 tumor growth in these mice. Significant tumor growth inhibition was also observed in the therapeutic setting. It was also shown that neoantigen-reactive CD8 T cells and IFN- γ expression increased within tumors after vaccination. Castle et al. also demonstrated tumor regression in C57BL/6 mice, this time inducing tumors with the B16F10 melanoma cell line.⁴² Survival was also examined, with 40% of the mice

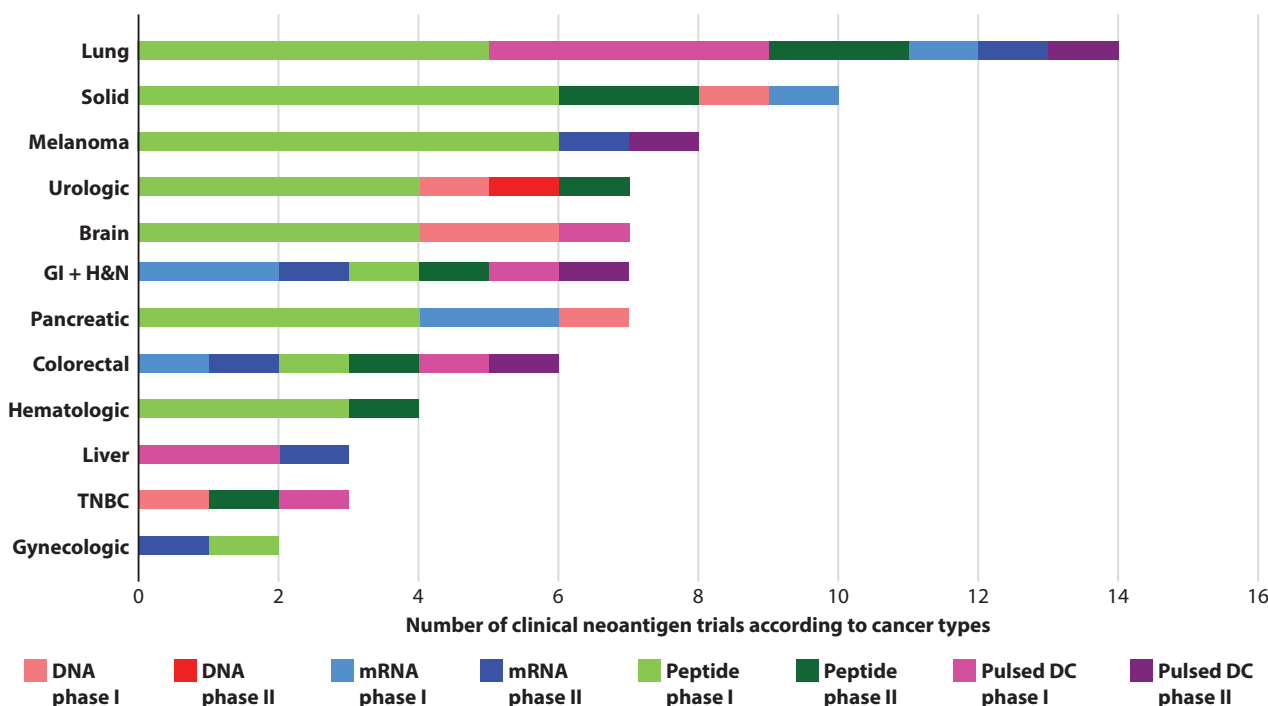


Figure 4. Clinical neoantigen trials according to cancer types. X-axis indicates the number of current, ongoing trials. Coloring indicates both the vaccine formulation and the status of the trial.

immunized with a particular neoantigen (“mut30”) surviving, while all mice within the control-treatment group died within 44 days. Numerous other examples of neoantigen-related tumor regression and enhanced survival in mouse models are available.^{27,43}

Clinical Studies

Given clear positive outcomes in animal models, a large number of human clinical trials are currently ongoing. Registered “neoantigen” clinical trials in www.clinicaltrials.gov, updated April 2020, and sorted by cancer types, experimental phases, and delivery platforms, are illustrated in **Figure 4**. Of 59 trials, 46 trials are in phase I, while 13 trials have proceeded to phase II. Breaking the trials down by treatment modality, 35 utilize neoantigen peptides, 11 involve neoantigen priming of DCs, and 13 use DNA or mRNA-based vaccines. Studies focus on cancers with known high neoantigen loads, such as non-small cell lung cancer, melanoma, and bladder cancer.

Several recently completed clinical trials have shown promising results. Yong Fang et al. revealed that peptide-based vaccines utilizing a total of 10 to 20 different synthetic long peptides per patient, were safe and elicited effective T cell responses in multiple advanced cancers such as non-small cell lung cancer, pancreatic cancer, colorectal cancer, melanoma, ovarian cancer (trial: NCT03662815). In post-vaccination blood samples, 80% of patients had significant T cell responses as detected by ELISpot assays (unpublished abstract: https://cancerres.aacrjournals.org/content/79/13_Supplement/CT006).

In another phase 1b trial, peptide-based vaccines were introduced in combination with nivolumab to 10 metastatic melanoma patients (NCT02897765). Siwen Hu-Lieskovan et al. reported that T cell responses in post-vaccination blood samples were detectable in 12 out of 14 peptide treatments. The responses were durable up to 52 weeks after the first dose, with significant changes seen in TCR repertoire analysis (unpublished abstract: https://cancerres.aacrjournals.org/content/79/13_Supplement/4096) Song Gao et al. report similar results in the case of a Chinese patient with chemo-resistant small cell lung carcinoma (SCLC) with brain metastasis. The patient received autologous DC-based neoantigen cancer vaccines. After 10 months, all brain metastases regressed. ELISpot assays of post-vaccination blood samples revealed detectable CD4 and CD8 responses to 8 out of 12 neo-epitopes (unpublished abstract: https://cancerres.aacrjournals.org/content/79/13_Supplement/942).

Therapies combined with vaccines

In the early stages of cancer, one modality of treatment, such as surgery, may possibly cure the disease. However, advanced cancers require multi-therapeutic agents such as chemotherapeutic, targeted, and immunotherapeutic drugs.⁴⁴ In current clinical trials, personalized cancer vaccines are often combined with additional agents to maximize the potential to improve clinical outcomes.⁴⁵ Supplementary agents used with personalized cancer vaccines in current clinical trials are seen in **Table 1**. Out of 59 trials, 28 combine neoantigen vaccines with either

Table 1. The agents currently used in combination with neoantigen therapy trials.

Groups	Subgroups	Drugs	Number of trials
Monoclonal antibody drugs	Checkpoint inhibitors: Anti-PD-1	Nivolumab	9
		Pembrolizumab	5
		Toripalimab	1
	Checkpoint inhibitors: Anti-PD-L1	Durvalumab	3
		Atezolizumab	4
	Checkpoint inhibitors: Anti-CTLA-4	Ipilimumab	8
		Tremelimumab	1
Anti-CD20	Rituximab	1	
Anti-VEGF	Bevacizumab	1	
Chemotherapy drugs	Anti-metabolites	Gemcitabine	2
		5-Fluorouracil	1
		Pemetrexed	1
	Alkylating agents	Carboplatin	2
		Oxaliplatin	1
		Cyclophosphamide	2
		Temozolomide	1
	Plant alkaloids	Nab-paclitaxel	1
		Irinotecan	1

immune checkpoint inhibitors, targeted therapies, or chemotherapeutic drugs, while 31 trials focus on neoantigen vaccines alone in order to evaluate safety and efficacy. In 12 trials, vaccines are coupled with more than one additional agent.

The most commonly used immune checkpoint inhibitors in clinical studies are anti-PD-1 agents (nivolumab and pembrolizumab), anti-PD-L1 (atezolizumab and durvalumab), and anti-CTLA-4 (ipilimumab). Immune checkpoint inhibitors are aimed at boosting T cell levels that have been exhausted in the tumor microenvironment.⁴⁶ Combining vaccines with immune checkpoint inhibitors have shown promising results in both pre-clinical and clinical trials.⁴⁷⁻⁴⁹ A phase 2 clinical trial suggests that the combination of peptide-based vaccines with nivolumab significantly improves overall survival in patients with HPV-driven cancers.⁴⁸ Not surprisingly, the response to immune checkpoint inhibitors is also determined by the mutational landscape; patients with high-tumor mutation burden (TMB) tend to have robust T cell reactivity and better clinical outcomes after receiving immune checkpoint inhibitors.^{50,51}

Chemotherapeutic drugs are believed to promote tumor immunity in several ways. Immunogenic cancer cell death activates both the innate and adaptive immune responses via dsDNA and cancer antigen release, respectively.⁵² Moreover, chemotherapy is capable of modulating immunorecognition via various mechanisms, such as the upregulation of co-stimulatory molecules and downregulation of various immune checkpoint molecules. There is no significant preference for particular chemotherapeutic drugs used in the trials. Examples are gemcitabine, carboplatin, and cyclophosphamide.

Platform comparison

Although putative neoantigens may be accurately predicted, selected, and validated, directing those epitopes to the proper immune cells to elicit tumor-specific CD4+ and CD8+ responses is also essential.⁵³ DNA-, RNA-, synthetic long peptide-, and

dendritic cell-based vaccines constitute the delivery platforms used in current clinical trials. **Table 2** allows comparison of the underlying platform mechanisms, formulations, advantages, and disadvantages. Amongst the four, peptide-based vaccines are found in the plurality of clinical trials. Here, polyinosinic-polycytidylic acid and poly-L-lysine (poly-ICLC), is the most widely-used adjuvant for peptide vaccines. Such treatment mimics dsRNA, a product of various viral infections, to activate TLR3.⁵⁴ The immune response driven by peptide vaccines is currently unpredictable at best, and often weak or temporary,⁵⁵ and thus requires the addition of adjuvants. DNA and mRNA platforms are believed to be inherently more immunogenic as these molecules naturally provoke the innate immune system,^{56,57} however, incorporation of modified nucleoside into these molecules, such as pseudouridine for mRNA, can help balance immune activation versus antigen stability and expression.⁵⁸ DNA vaccines cloned into a plasmid are designed for host nuclear retention without integration into the host genome, while mRNA is directed to the cytoplasm for translation.⁵⁹ However, mRNA can easily be degraded by extracellular RNases. As a result, protective strategies, such as liposome- and nanoparticle-encasement, are essential for efficient RNA delivery.⁵⁷ Additionally, lipid nanoparticle encased pseudouridylated mRNA has been shown to have an adjuvant effect, stimulating both T helper and germinal B cells.⁵⁸ Given these potentially strong immune responses as well as a low risk of genomic integration, mRNA-based vaccines are becoming increasingly popular, with a number of prominent biotech companies favoring this approach. In collaboration with BioNTech, Sahin's group has been assessing safety and efficacy of TAA-based and TSA-based mRNA vaccines in more than 30 TNBC patients (NCT02316457). Moderna, a biotech whose rapid development of a potential Covid-19 vaccine has made headlines, has led a clinical trial introducing mRNA vaccines with and without pembrolizumab to 33 resected solid

Table 2. The platforms by which neoantigens may be delivered.

Platform	Mechanisms	Formulations	Pros	Cons	Clinical Trial Example
DNA	- STING/TBK1/IRF3 - TLR 9	Cloned plasmid	- Amplification to multiple peptides - Polypeptides in a plasmid available	- Possibly integrates into genome - Production involves bacteria	- NCT03122106 (phase I ongoing) - NCT03199040 (phase I ongoing)
mRNA	- TLR 3, 7, 8	- Naked - Liposomes - Nanoparticles - Viruses	- Do not integrate into genome - Polypeptides in an mRNA available - mRNA may have "self-adjuvant" effects	- Instability: protection from RNases required - In vitro transcription: expensive	- NCT03908671 (phase I ongoing) - NCT03480152 (phase I: no "serious adverse events"; phase II: ongoing)
Peptide	- Internalization by DCs - Cross-presentation under appropriate activation and processing	Synthetic long peptide with poly-ICLC	- Stable - Safe - More data from clinical trials	- Low immunogenicity - Multiple epitope production required - Adjuvant required	- NCT03645148 (phase I ongoing) - NCT02950766 (phase I ongoing)
DC	- Antigen presentation to T cells	Mature and activated DCs	- Possibly the best way to prime CD8 and CD4 T cells	- Expensive - Time-consuming - In vitro peptide loading required	- NCT04105582 (phase I ongoing) - NCT04078269 (phase I ongoing)

tumor patients (NCT03313778). Clinical outcomes and T-cell responses were sufficient to proceed to a phase-2 trial on high-risk melanoma patients (NCT03897881). Intuitively, DC-primed vaccines might offer the optimal strategy to initiate T cell responses, bypassing steps from DNA transcription to peptide presentation. Not surprisingly though, cost and time constraints may significantly limit the benefits of such an approach.⁵⁵ Hopefully, the current generation of clinical trials will generate clarity as to the optimal platforms for particular cancers.

Limitations of neoantigen-based therapy

We'd be remiss in failing to mention possible stumbling blocks. As seen above, identification of neoantigens remains a fluid area of research, with little consensus as to the neoantigen sources that are most immunogenic, while neoantigen combination therapies and delivery methods are far from optimized. Additionally, regulatory hurdles are substantial; current practices are centered on drugs with specific molecular formulas that have undergone years of testing. Such a paradigm obviously cannot be applied to neoantigen development, where personalized treatments must be generated in a matter of weeks.⁶⁰

At the biological level, a number of studies suggest that many immunogenic neoantigen candidates are passengers, not drivers of cancer development.⁶¹ Additionally, driver mutations may be selected for weak immunogenicity.⁶² The heterogeneity of cancer tissue means that even strongly immunogenic neoantigens may fail to elicit whole tumor destruction. Also, loss of HLA heterozygosity (HLA-LOH) is a common immune-evasion tactic in cancer. In one study, loss of HLA was seen in 40% of non-small-lung cell cancer samples,⁶³ eliminating some degree of neoantigen presentation capacity. Not surprisingly, however, organisms do not necessarily take kindly to HLA-LOH, which may stimulate the activity of NK cells.⁶⁴ More often, it has been argued, HLA is overexpressed in cancer.⁶⁵

Conclusions

We've highlighted sources of neoantigens and the status of clinical trials as particularly fast-moving subjects. A number of other topics deserve at least a mention. First, a glimpse at these clinical trials shows that neoantigen-therapies are predominantly administered in combination with immunomodulatory drugs. While PD-1/PD-L1 inhibitors are currently dominant, numerous other immuno-inhibitory molecules (e.g. TIM-3, VISTA) and stimulatory molecules (e.g. OX40, CD40) are currently being targeted by next-generation drugs.⁶⁶ Thus optimization of therapy combinations remains a fluid area of research. Secondly, while it is generally thought that neoantigen immunogenicity correlates with HLA-binding affinity,⁶⁷ there are some indications that poorly-binding neoantigens may still elicit immune responses.^{68,69} Delineation of the precise neoantigen characteristics that elicit immunogenicity will likely reveal nuance. Finally, we should point out that there is no reason that cancer-unique antigens must be confined to peptides. Some HLA variants (e.g. HLA-E, MR1) are capable of presenting lipids and metabolites, some of which may be unique to cancers and/or infections.⁷⁰

Practically speaking, the expenses involved in pipelines that require multiple "omics" protocols, the rapid output of peptides or peptide-coding RNA/DNA vectors, and treatment with complementary immunotherapeutics, cannot be denied. It is comforting to note, however, that labs around the world are working diligently to minimize such costs, emphasizing compassion over profit.

Competing interests

The authors declare no conflicts of interest.

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