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Personalized cancer vaccines: targeting the cancer mutanome

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Abstract

The development of next generation sequencing technologies has revolutionized our understanding of how specific genetic events contribute to cancer initiation and progression. Dramatic improvements in instrument design and efficiency, combined with significant cost reductions has permitted a systematic analysis of the mutational landscape in a variety of cancer types. At the same time, a detailed map of the cancer mutanome in individual cancers offers a unique opportunity to develop personalized cancer vaccine strategies targeting neoantigens. Recent studies in both preclinical models and human cancer patients demonstrate that neoantigens (1) are important targets following checkpoint inhibition therapy, (2) have been identified as the target of adoptive T cell therapies, and (3) can be successfully targeted with personalized vaccines. Taken together, these observations provide strong rationale for the clinical translation of personalized cancer vaccines.

INTRODUCTION

It has long been known that solid tumors can elicit antitumor immune responses, often preventing the outgrowth of early lesions [1–6]. The tumor immunosurveillance concept was dramatically extended by Schreiber and colleagues to more accurately describe the dynamic interplay between tumors and the immune system. Cancer immunoediting is a dynamic process that describes the outgrowth of clinically apparent disease in immunocompetent hosts in three sequential phases: elimination, equilibrium, and escape [4]. Of particular note to the field of cancer immunotherapy is the observation that even in the escape phase tumor cells express antigens that can elicit antitumor immunity. Thus, great emphasis has been placed on identification of relevant tumor antigens and strategies to induce and/or enhance antitumor immunity.

For many years the identity of tumor antigens was only sporadically known. However, in 1991 Boon and colleagues introduced a molecular strategy to identify tumor antigens

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CONFLICT OF INTEREST

The authors declare that no conflicts of interest exist.

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recognized by T cells [7]. Subsequent studies demonstrated that tumor antigens include both self and nonself antigens. Typically self tumor antigens (tumor-associated antigens) are derived from normal proteins with high expression in the tumor but limited expression in normal tissues. Nonself tumor antigens include both neoantigens and viral antigens. While both types of tumor antigens have been implicated in tumor development and progression, they are distinct from an immunological perspective. Tumor-specific antigens are expressed only in the tumor and immune therapies targeting such antigens are less likely to be associated with autoimmunity, and the repertoire of responding T cells is less likely to have been shaped by thymic selection (Figure 1). This suggests that the affinity of responding T cells may be higher.

Human neoantigens have been identified in a number of different tumor types over the past 20 years (Reviewed in [8]). Recently, the systematic identification of neoantigens has been dramatically simplified through next-generation sequencing technologies whereby entire cancer exomes (and comparative normal exomes) can be sequenced and compared. The progress in high-throughput sequencing at deep coverage has revolutionized the path towards personalized medicine, and provides unique opportunities to pursue the development of personalized cancer vaccines. At the same time, results from the first clinical studies of checkpoint inhibition using antibodies targeting CTLA4, PD-1, and PDL1 have shown promise and provide proof-of-concept that T cell-based immunotherapy can offer therapeutic benefit in a variety of tumors. We discuss here current strategies for the discovery and validation of neoantigens, and the clinical implications for vaccine development.

NEXT GENERATION SEQUENCING AND IDENTIFICATION OF NEOANTIGENS

Massively parallel or next generation sequencing technologies have transformed nucleic acid sequencing, significantly decreasing the cost and time required to sequence human genomes [9]. In the field of cancer genomics, the realization that somatic mutations present in cancers contribute to cancer initiation and progression has spurred efforts to sequence cancers. Cancer genome sequencing rapidly progressed from patients with hematologic malignancies in 2008 [10] to solid malignancies with more complex mutational landscapes such as breast cancer [11]. Since then, cancer sequencing studies have been performed in multiple different types of cancer, with the largest effort being through The Cancer Genome Atlas (TCGA), a program launched in 2006 by the NIH and NHGRI to comprehensively characterize the genomic and molecular features of various cancer types. In order to define the somatic mutations present in the tumor, it is necessary to compare tumor and germline DNA. To confirm that the mutations are expressed, tumor RNA is required. For whole exome sequencing, and in particular RNA sequencing, a high quality sample is critical. Traditionally tissue obtained at surgery has been used. Currently, adequate DNA/RNA can often be obtained from fresh and/or paraffin-embedded core biopsy samples. Through TCGA, exome or whole-genome sequencing has been completed for close to 30 different cancer types [12]. Similar efforts are ongoing in other countries such as the UK [13]. These efforts also include detailed analyses of the molecular subtypes of cancers such as breast and

pancreas [14, 15]. Of note, the mutational landscape varies considerably among the different histologic types of cancer; analysis of 27 cancer types showed that the median frequency of somatic mutations in cancers ranged from 0.1/megabase (approximately one mutation in the entire exome) in pediatric tumors to over 100/megabase in lung cancer and melanoma [12]. To facilitate analysis of sequencing results, novel bioinformatics programs have been developed in parallel to analyze and interpret the vast amount of data obtained. Together, these technologies are now being used to explore the potential of personalized therapeutics. The recently announced NCI-MATCH (Molecular Analysis for Therapy Choice, www.cancer.gov/nci-match) trial will use next generation sequencing technologies to identify the mutations present in individual patient's cancers, and then use this information to treat patients with molecularly targeted cancer drugs specifically designed to target the mutations present [16].

An equally exciting therapeutic opportunity is the design of personalized vaccines based on the identification of mutations present in individual patient's cancers. Of all the genetic changes that have been observed in the cancer genome, single nucleotide variations (SNVs) that encode missense mutations have the highest likelihood of being expressed and recognized by the immune system. In a recent study of 47 luminal breast cancer genomes, approximately 31 nonsynonymous SNV mutations were present in each breast cancer genome, similar to the number identified in previous studies of a basal-like breast cancer [11], and similar to the number predicted by limited exome sequencing [17], and statistical models [18]. The question as to whether somatic mutations can be processed and presented by the immune system was first addressed by *in silico* analysis of the consensus coding sequences of human breast and colon carcinoma [19]. Using computer algorithms, the authors analyzed data from limited exome sequencing studies of breast and colorectal cancer to determine how many mutations detected by sequencing could be meaningfully presented by HLA-A2, the most common HLA class I allele. In these studies, Segal et al. predicted that approximately one new HLA-A2-restricted epitope was generated for every 10 mutations identified by sequencing [19], and individual breast cancers accumulate approximately 10 novel HLA-A2 epitopes. As individual tumors can express up to six distinct HLA class I alleles, the estimated number of novel epitopes may be significantly greater than that predicted for a single HLA allele alone.

NEOANTIGENS CAN BE RECOGNIZED BY THE IMMUNE SYSTEM

Even before the advent of next generation sequencing, sporadic reports documented that human neoantigens can be recognized and/or targeted by the immune system. Sensi et al. demonstrated *in vitro* recognition of neoantigens using autologous tumor-specific T cells from peripheral blood and tumor [20]. Early stage human clinical trials targeting mutant p53 or KRAS demonstrated that patients who developed T cell responses had a significantly improved survival [21]. Similarly, patients being treated with adoptive cell therapy with T cells specific for neoantigens have been reported to have clinical responses [20].

Next-generation sequencing now provides robust technologies to identify genetic changes and candidate antigens (Figure 2). As several thousand mutations may be present in an individual cancer, algorithms are required to prioritize mutations for immune targeting. The

first step to prioritize neoantigens is to confirm expression of the mutant genes in the tumor. Up to 40% of the mutant genes identified by exome sequencing are not expressed due to early termination of transcripts or other reasons. Mutant gene expression can be confirmed by sequencing tumor mRNA (RNA-Seq or RT-PCR using cDNA from tumor RNA). The second step to prioritize neoantigens is to estimate potential binding to the appropriate HLA alleles. For this, computer-based algorithms that quantitate the predicted binding affinity of candidate epitopes to class I HLA alleles (<http://www.iedb.org/>) [22] can be used. Predicted binding can be validated by *in vitro* binding studies in which synthetic peptides corresponding to the candidate neoantigens are tested for binding to the HLA allele of interest. Additional algorithms can be incorporated that quantitate how well candidate neoantigens are processed by the proteasome and transported into the endoplasmic reticulum. Of note, an *in silico* exome-based epitope prediction algorithm was recently tested using a data set of 17 previously identified human neoantigens. Of these 17 antigens, 14 passed the cut-off for binding to HLA alleles and 15 passed the cut-off for antigen processing, confirming the value of current computational epitope identification pipelines [23].

It should be noted that HLA class I molecules play a key role in the presentation of tumor antigens and thereby in the success of T cell-based immunotherapy. Loss or reduced expression of HLA class I molecules by tumor cells has been reported in a number of cancers, and is associated with reduced levels of tumor infiltrating T cells [24] and immune escape [25, 26]. To counteract reduced expression of HLA class I molecules, pro-inflammatory cytokines, such as interferons or IL-12, can be used to upregulate HLA class I molecules and antigen presentation.

One of the first preclinical studies to apply a systematic approach to cancer exome analysis and T cell epitope prediction was published in 2012 by Matsushita et al. [27]. In their study, next generation sequencing was used to identify the dominant rejection antigens present in two highly immunogenic, unedited methylcholanthrene (MCA)-induced sarcoma cell lines, d42m1 and H31m1. Both of these MCA sarcomas grow progressively in immune deficient mice, but are rejected when transplanted into wild type mice. The investigators identified 3398 nonsynonymous missense mutations in d42m1, and 2391 nonsynonymous missense mutations in H31m1 by cDNA capture sequencing. Using a MHC class I algorithm to assess potential binding of candidate neoantigens to MHC class I alleles, they identified mutant spectrin- β 2 as the top candidate rejection antigen in d42m1. Functional analyses demonstrated that mutant spectrin- β 2-specific T cells are present in d42m1 tumors at the time of rejection, and tumor cell clones lacking mutant spectrin- β 2 grow aggressively in wild type mice. These data confirm that neoantigens are an important target during cancer immunoediting, and established the initial proof-of concept that next generation sequencing and epitope prediction algorithms can be used to identify and prioritize candidate neoantigens.

Sahin et al. made similar observations in the B16F10 melanoma model. They demonstrated that sequencing-identified neoantigens present in B16F10 can be recognized by the immune system [28]. Of 962 nonsynonymous mutations identified, 11 of the prioritized candidate antigens were able to induce T cell responses following vaccination without cross-reactivity

against the corresponding wild type epitope. Vaccination with selected prioritized candidate antigens was also able to protect mice from tumor challenge. Parallel studies in humans by two independent groups have demonstrated that neoantigens can be identified using exome sequencing [29, 30]. Both groups performed exome sequencing of tumor tissue from melanoma patients and identified neoantigens recognized by tumor infiltrating lymphocytes (TILs). Taken together, these reports confirm the feasibility of exome sequencing for neoantigen identification in human cancers and provides strong rationale for development of personalized cancer immunotherapies.

TARGETING NEOANTIGENS

Personalized vaccines targeting neoantigens have significant theoretical advantages. Recent studies have explored the potential of personalized cancer vaccines in preclinical models. Studies in several tumor types confirm that both CD4 and CD8 T cell responses to neoantigens can be successfully induced. In their study of B16F10 melanoma, Sahin et al. demonstrated that they could successfully generate immune responses to 16 of 50 prioritized neoantigens using a synthetic peptide vaccine platform [28]. Vaccines corresponding to two of these prioritized neoantigens were further tested for treatment of B16F10 tumor-bearing mice. In both cases, synthetic long peptide vaccines were able to significantly inhibit tumor growth. In follow-up studies, the authors expanded on these findings by demonstrating that polyepitope mRNA vaccines targeting sequencing-identified neoantigens in three mouse tumor models induced potent tumor-specific immune responses and tumor protection. Of note, while the tumors did not express detectable levels of MHC class II molecules, the majority of immunogenic neoantigens induced CD4 rather than CD8 T cell responses. Tumor control was dependent on CD40 ligation and CD8 T cells [31].

In collaboration with the Schreiber Laboratory, we recently demonstrated that neoantigens are important targets for CD8 T cells following checkpoint inhibition immunotherapy in a murine sarcoma model [32]. We refined our initial next generation sequencing and epitope prediction algorithms for the identification and prioritization of neoantigens presented by MHC class I. Rather than relying on a single computational algorithm to predict binding of candidate epitopes to MHC alleles, we used a combination of three different algorithms and computed a median binding score. Expression of the top two candidate neoantigens in the context of MHC class I molecules on the surface of tumor cells was confirmed using immunoproteomics. Synthetic long-peptide vaccines incorporating the two immunodominant neoantigens (mLama4 and mAlg8) induced CD8 T cell-dependent tumor rejection in both prophylactic and therapeutic settings. Other investigators have used a similar strategy in two commonly studied mouse tumor models, MC-38 (colon cancer) and TRAMP-C1 (prostate cancer) [33]. Using a combination of whole exome and transcriptome sequencing, the number of predicted MHC class I-binding neoantigens differed by almost 10-fold (170 vs 6 for MC-38 and TRAMP-C1, respectively). Immunoproteomics reduced the number of candidates in MC-38 to 7 while no candidates were identified in TRAMP-C1. Subsequent prediction analysis for binding to MHC class I alleles and recognition by T cells, combined with peptide vaccine studies further reduced the number of neoantigens capable of conferring tumor protection.

Together, these preclinical studies are encouraging as they provide proof-of-concept that neoantigens can be identified and prioritized using well defined algorithms, and then successfully targeted with vaccines. In particular, the combination of checkpoint inhibition and personalized vaccine therapy may enhance the immune system's ability to eliminate tumors by promoting responses to both dominant and subdominant antigens. In addition, it is possible that vaccination to neoantigens will trigger epitope spreading, as has been observed with vaccines to tumor-associated antigens [34, 35]. Of note, whereas the total number of nonsynonymous identified in individual tumors can be as high as several thousand, the final number of validated neoantigens is often much lower, and none may be present in some tumors, as was found for the TRAMP-C1 prostate tumor [33]. Thus, while the process by which mutations are prioritized has demonstrated to be effective in multiple cases, there is still room for improvement.

IMPLICATIONS AND ONGOING CLINICAL TRIALS

Prioritized neoantigens can be targeted using personalized vaccine approaches. To date most cancer vaccines have been tested in patients with locally advanced or metastatic disease. However, this may not be the ideal clinical context, as regulatory networks limit antitumor immune responses in this context. Vaccination in patients with no or minimal disease such as after surgical resection to prevent recurrence is believed to be an ideal clinical scenario for vaccine efficacy. For personalized cancer vaccines, tumor tissue is required to identify and prioritize candidate neoantigens, and coordination of tissue acquisition, vaccine construction, and vaccine administration is essential (Figure 2). In addition, integration of standard therapies and/or additional immune-based therapy such as checkpoint inhibition can further complicate vaccine treatment paradigms. We are performing initial phase 1 clinical trials in breast cancer patients with locally advanced triple negative breast cancer with persistent disease following neoadjuvant chemotherapy (NCT02348320 and NCT02427581, Figure 3). These patients have a high risk of disease recurrence, but there is a window of opportunity to administer personalized vaccines following standard of care therapy. The continuing improvements in sequencing efficiency and turnaround time will likely permit the integration of personalized vaccine therapy in an increasing number of clinical scenarios.

As with cancer vaccines that target tumor-associated antigens, multiple vaccine platforms are being explored for personalized cancer vaccines (Table 1). Personalized vaccines do have particular manufacturing requirements, and must have the manufacturing flexibility to rapidly integrate the neoantigens of interest. DNA vaccines represent a flexible and robust platform, with documented safety and efficacy in clinical trials [36]. An attractive attribute of DNA vaccines is the opportunity to genetically modify the targeted antigens as well as integrate additional genes such as those encoding immunomodulatory proteins or cytokines. The molecular flexibility of the DNA vaccine platform allows targeting multiple neoantigens using a single polypeptide DNA vaccine. Rather than encoding the entire mutant gene, the recombinant construct encodes fragments of the mutant genes to include the individual mutations and 10–15 flanking amino acid residues [9].

An alternative approach to target neoantigens is through peptide-based vaccines. Peptide-based cancer vaccines have been investigated extensively ever since the first human tumor antigen was identified in 1991 [7]. First generation peptide-based cancer vaccines have typically included “minimal epitope” peptides, i.e. peptides of 9–10 amino acids in length known to bind to specific MHC class I alleles. First generation peptide-based cancer vaccines have an excellent safety profile, and have been successful in inducing peptide-specific T cell responses in the peripheral blood of cancer patients, as measured by ELISPOT analysis or tetramer analysis. In addition, clinical responses have been detected, such as with the E75 peptide, a HER2/neu-derived minimal peptide known to bind to HLA-A2 [37]. The encouraging results with first generation peptide-based cancer vaccines prompted investigators to explore improvements to the platform, and have resulted in “next-generation” peptide-based cancer vaccines [38]. One strategy that is particularly promising is the use of synthetic long peptides that are 25–30 amino acids in length [39]. Due to their longer length, synthetic long peptides are processed by the immune system differently than minimal epitope peptides, requiring uptake and processing by professional antigen-presenting cells (APC) such as dendritic cells. This requirement for uptake and processing by professional APC appears to circumvent the transient T cell induction that may occur when minimal epitope peptides bind to MHC molecules on non-professional APC [39]. An additional advantage of the requirement for uptake and processing by professional APC is that synthetic long peptides appear to prolong the duration of peptide presentation, and T cell activation [40]. Processing of synthetic long peptides and T cell activation can be further enhanced through the activation of toll-like receptors on the surface of APC. In particular, the TLR3 agonist, poly IC:LC has been used as an adjuvant to enhance the efficacy of synthetic long peptide-based vaccines targeting tumor-associated antigens [41–43]. In two ongoing phase 1 clinical trials of personalized cancer vaccines, synthetic long peptides targeting neoantigens are being tested in patients with malignant melanoma (NCT01970358) and glioblastoma (NCT02287428), but no results have been reported to date. We have initiated a personalized synthetic long peptide vaccine trial in patients with persistent triple-negative breast cancer following neoadjuvant chemotherapy (NCT02427581).

Dendritic cells represent a third potential platform for personalized vaccines. Dendritic cells can be expanded and matured *ex vivo*, and these cells can be pulsed with peptides corresponding to the neoantigens prior to vaccination. This strategy was used successfully by Carreno et al. in melanoma [44]. Neoantigens were identified and prioritized by exome and cDNA capture sequencing. Administration of personalized dendritic cell vaccines elicited significant CD8 T cell immune responses in patients. Three patients with stage III melanoma who received prior anti-CTLA4 (ipilimumab) therapy were selected. Seven candidate neoantigens were incorporated into the vaccine together with two gp100-derived peptides. In all patients, the vaccine significantly increased the CD8 T cell response to one of the targeted antigens. Vaccination against the neoantigens was found to be safe, and none of the patients had evidence of autoimmunity. This is the first report to date of results from a phase 1 clinical trial of a personalized cancer vaccine.

While patients and investigators anxiously await the results of these first generation personalized cancer vaccine trials, second generation personalized cancer vaccine strategies are in development. The recent success of checkpoint inhibition using monoclonal

antibodies targeting CTLA4 [45–47], PD1 [46, 48, 49] or PD-L1 [50, 51] suggests that the combination of checkpoint inhibition and personalized cancer vaccine therapy should be explored [52–54]. Looking forward, if preliminary studies demonstrate that personalized vaccines can elicit tumor-specific immune responses, this would open the door to explore a large number of potentially synergistic combination strategies including vascular reprogramming, reversal of T_{reg} and/or MDSC, and checkpoint inhibition. For an in-depth discussion of immunotherapy-based combination strategies we refer to the elegant review by Chen and Mellman [54] in which the authors introduce seven discrete steps in the “cancer-immunity cycle.” Each step is modulated by stimulatory and inhibitory factors, and the authors highlight examples of strategies that might affect these steps. In summary, immune targeting of the cancer mutanome is an area of intense research interest and great promise.

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Research highlights

- Next generation sequencing technologies can identify candidate neoantigens in different types of cancers
- Neoantigens can be recognized by the immune system
- Neoantigens can be successfully targeted by personalized vaccines

Figure 1. Neoantigen presentation

Schematic representation of tumor-associated and neoantigen presentation.

Figure 2. Overview of personalized cancer vaccine design

Patient tumor tissue and PBMC are subjected to exome and RNA sequencing to identify and confirm expression of nonsynonymous somatic mutations. Validated mutations are pipelined into MHC class I epitope prediction algorithms to prioritize a list of candidate antigens which are further prioritized by IFN- γ ELISPOT and peptide binding assays. Personalized vaccines targeting validated neoantigens are synthesized under GMP conditions and administered following standard of care therapy.

Figure 3. Design of phase 1 clinical trial of a personalized breast cancer vaccine strategy

Patients diagnosed with newly diagnosed clinical stage II/III triple negative breast cancer receive standard of care therapy including neoadjuvant chemotherapy, surgery, and radiation therapy. Tumor tissue and PBMC are obtained at the time of diagnosis. Tumor tissue and PBMC are sequenced to identify nonsynonymous somatic mutations expressed in the tumor. These mutations are pipelined into MHC class I epitope prediction algorithms to prioritize a list of candidate antigens which are validated *in vitro*. Validated neoantigens are integrated into a DNA vaccine that is synthesized under GMP conditions. The vaccine is administered following standard of care therapy.

Table 1

Salient features of various platforms available for developing personalized vaccines against tumors.

Vaccine Platform	Advantages	Challenges/Limitations
Synthetic peptides	<ul style="list-style-type: none"> • Easy manufacturing and storage • Robust T cell response • Restriction to minimum required epitope limits autoimmunity 	<ul style="list-style-type: none"> • Tolerance with short peptides • Limited tumor rejection/growth arrest • Multiple peptides need to be incorporated to improve efficacy
DNA vaccine	<ul style="list-style-type: none"> • Flexible platform • Rapid cloning, isolation and upscaling of production • Adjuvants can be co-expressed with antigen • Can be engineered to alter the intracellular routing of antigen 	<ul style="list-style-type: none"> • Suboptimal immunogenicity in humans • High transfection rates required
Dendritic Cell vaccine	<ul style="list-style-type: none"> • Multiple and unknown antigens covered at once • Polyclonal response • Activation of both innate and adaptive immunity • Patient specific (autologous cell based) 	<ul style="list-style-type: none"> • Time intensive and expensive to produce • Difficult to characterize specificity and modulate response • Poor immunogenicity of tumor cells
Viral vectors	<ul style="list-style-type: none"> • Allows insertion of multiple transgenes • Localized inflammation promotes vaccine uptake and efficacy 	<ul style="list-style-type: none"> • Pre-existing immunity against vectors may compromise efficacy • Insertional mutagenesis • Boost strategies remain challenging