Tumor neoantigens: building a framework for personalized cancer immunotherapy

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Introduction

The idea that tumors of nonviral origin possess unique, tumor-specific antigens (TSAs) arose from work reported in the first half of the twentieth century by several groups, particularly those led by Gross, Foley, Prehn, and Old (1–4). These studies showed that when inbred mice bearing carcinogen-induced tumors were cured by surgical resection, they were immune to subsequent rechallenge with the same tumor cells, but not with other distinct tumor cells, even those derived in the same manner from different hosts. In the ensuing next quarter century, the roles of the MHC proteins in antigen presentation were discovered (5, 6), methods were developed to propagate antigen-specific cytolytic T lymphocytes (CTL) in culture (7, 8), and it became possible to clone and express gene products using molecular biology techniques. Together, these developments provided the elements needed for the first molecular identification of TSAs. Using mutagenized forms of preexisting P815 mastocytoma tumor cells, Boon and colleagues isolated a highly immunogenic, nontumorigenic tumor cell variant (tum–) and used CTL-based expression cloning approaches to show that a point mutation in a ubiquitously expressed protein (P91A) was responsible for the immune rejection of these tumor cells in naive, syngeneic WT mice (9). Shortly thereafter, Hans Schreiber and colleagues showed that tumor-specific mutant proteins could also function as tumor neoantigens in a host genome comprised both tumor-specific and tumor-associated antigens (TAAs).

During this time, independent efforts by Knuth and Old and the Rosenberg group identified T cells in the peripheral blood and tumors of melanoma patients that predominately reacted with melanoma but not normal cells, suggesting that human cancers also possessed tumor antigens whose expression was either tumor specific or showed limited expression in normal cells (12, 13). In 1991, Boon and colleagues used their T cell–based approach to clone the first human tumor antigen (MAGEA1) (14), then subsequently cloned a range of different human tumor antigens that included, among others, those derived from tumor-specific mutant genes, alternatively initiated proteins, normal proteins that displayed aberrant quantitative or qualitative expression in tumor cells, and proteins expressed only in germ cells and tumor cells (15). At about the same time, Sahin et al. used an autologous antibody-based cloning approach (SEREX) and also identified different types of human tumor antigens (16). Together, these pioneering efforts led to the acknowledgement that the tumor antigenome comprised both tumor-specific and tumor-associated antigens (TAAs).

Tumor antigen classification

Today we recognize three broad classifications of tumor antigens: TSAs, TAAs, and cancer-germline/cancer testis antigens (CTAs) (15, 17–19). TSAs are antigens that are not encoded in the normal host genome and may represent either oncogenic viral proteins or abnormal proteins that arise as a consequence of somatic mutations (i.e., neoantigens). During cancer initiation and progression, tumor cells acquire protein-altering mutations that are either responsible for transformation (driver mutations) or are byproduct of the genomic instability that accompanies cellular transformation (passenger mutations) (20–23). Some of these...
alterations may result in expression of mutant proteins that are perceived as foreign proteins by the immune system. This class of antigens is likely to be less susceptible to mechanisms of immunological tolerance and therefore may represent more visible targets for immune-mediated tumor control (19, 24). TAAs include proteins encoded in the normal genome and may be either normal differentiation antigens or aberrantly expressed normal proteins. Overexpressed normal proteins that possess growth/survival-promoting functions, such as Wilms tumor 1 (WT1) (25) or Her2/neu (26), represent TAAs that directly participate in the oncogenic process. Posttranslational modifications of proteins such as phosphorylation may also lead to formation of TAAs (27, 28). Because TAAs are normal proteins, their antigenicity depends on abnormal expression levels or context to circumvent naturally occurring mechanisms of immunological tolerance (29, 30). Along these lines, TAAs usually have lower T cell receptor (TCR) affinity compared with TSAs or foreign antigens (31). The third category comprises CTAs, which are normally expressed in testis, fetal ovaries, and trophoblasts, but can also be expressed in cancer cells (17). Because they are encoded in the normal genome but display highly restricted tissue expression, CTAs have received considerable attention as attractive targets for immunotherapy (32).

Paving the way for TSA-based cancer immunotherapy

In 2005, two important human studies stimulated increased interest in tumor neoantigens as therapeutic targets for cancer immunotherapy. First, using expression-cloning approaches, T. Wölfel et al. showed that the naturally occurring antitumor T cell response in a melanoma patient was directed toward neoantigens formed by somatic point mutations in five distinct genes and that T cell responses against these TSAs prevailed over responses to TAAs (33). Second, Rosenberg and Robbins showed that ex vivo–expanded tumor-infiltrating lymphocytes (TILs), when adoptively transferred into a melanoma patient who subsequently underwent a complete tumor regression, contained T cells that were specific for two mutant tumor antigens (34). T cells specific for these neoantigens persisted in the blood and tumor of the patient after adoptive transfer. Together, these data provided support for the concept that T cells recognizing tumor neoantigens could indeed provide substantial therapeutic benefit to human cancer patients.

These results came at a time when the majority of efforts in the field were focused on identifying TAAs and CTAs for use in cancer immunotherapy. During this period, limited effort was expended on TSAs because their identification was so difficult and represented a case of too much effort for too little gain for too few individuals. However, by 2008, cancer genome sequencing had clearly established that all cancers express somatic mutations (35, 36). These observations led Allison and Vogelstein to conduct in silico analyses of exome-sequencing data from breast and colorectal cancers, where they found several mutations that were predicted to form tumor-specific mutant antigens for CD8+ T cells (37), but it would take four years before experimental support could be obtained to corroborate their computational predictions.

In 2012, two independent reports provided the first unequivocal evidence that genomics and bioinformatics approaches could be used to identify tumor-specific mutant proteins that function as TSAs. Using a combination of next generation sequencing, in silico epitope prediction, and immunological approaches, Sahin and colleagues (38) and our group (39) independently identified and validated distinct TSAs in murine B16-F10 melanoma tumor cells and in highly immunogenic methylcholanthrene-induced (MCA-induced) sarcoma cells, respectively. Importantly, these studies showed that the time frame needed to identify TSAs could be shortened to only a few weeks as opposed to the months required using conventional antigen-cloning approaches. Moreover, our study (39) and an independent study by Dupage and Jacks (40) showed that TSAs were also key targets of cancer immunoediting — the immunological process that not only protects against cancer development, but also sculpts the immunogenicity of cancers that form in an immunocompetent individual (41–43).

The following year, this work was extended to human cancers. Robbins and Rosenberg showed that exome sequencing could be used to identify mutated human TSAs recognized by adoptively transferred tumor-reactive T cells (44). Concomitantly, Schumacher used a combination of exome sequencing and high-throughput MHC tetramer screening to show that checkpoint blockade immunotherapy facilitated expansion of preexisting T cells specific for tumor neoantigens in a human melanoma patient (45). Checkpoint blockade therapy, originally developed by Allison and colleagues, is based on the capacity of antibodies to block inhibitory receptors (such as cytotoxic T lymphocyte–associated protein 4 [CTLA-4]) on T cells or deplete inhibitory receptor–bearing T cells, thereby unleashing suppressed T cell–dependent antitumor effector functions in tumor-bearing hosts (46–48). The capacity of epitope prediction algorithms to identify human TSAs was further demonstrated in both prospective and retrospective analyses performed by Fritsch, Hacohen, and Wu (49, 50), Schumacher and colleagues (51), and Nelson et al. (52). Together, the mouse and human studies suggested that it was indeed possible to use genomics and bioinformatics approaches to rapidly identify mutant proteins expressed exclusively in cancer cells that function as tumor neoantigens.

Identifying tumor-specific mutations and predicting their capacity to function as TSAs

Whereas these initial studies revealed the power of combining genomics, bioinformatics, and immunological approaches to identify mutant TSAs, additional refinements have been made over the last few years that further improved the accuracy of the process.

Next generation sequencing. Advances in sequencing technology have transformed our ability to decode cancer-specific mutations by coupling the sequencing reaction with detection of nucleotide incorporation events for hundreds of millions of genomic fragments in the same instrument run (53). In particular, tumor-specific or “somatic” mutations can be identified using massively parallel sequencing (MPS) (17) approaches to compare DNA isolated from tumor versus normal sources. Similar to DNA-based assays using MPS, RNA from tumors can be analyzed by conversion to cDNA and construction of a library suitable for sequencing. Since the genome is large (3 billion base pairs) and its analysis complex, the advent of hybrid capture technology has permitted investigators to focus only on the 1% of the genome that comprises the coding exons of known genes, (i.e., the “exome”) (54–56). Here, probes designed to bind the exon sequences of annotated
genes are synthesized, biotinylated, and hybridized in solution with a fragmented whole genome library. The probe-bound library fragments are subsequently captured and isolated using streptavidin-coated magnetic beads. After release from the beads by denaturation, the library fragments are amplified, quantitated, and sequenced.

Exome-capture can be used in a clinical setting, but challenges include (a) obtaining information in a clinically relevant time frame, (b) the small amounts of DNA/RNA available from a core biopsy procedure, (c) tissue preservation in formalin and paraffin (formalin-fixed paraffin embedded [FFPE]), which promotes the degradation of nucleic acids via backbone crosslinking, and (d) data interpretation. Recent technical innovations have reduced the time for this approach from approximately one week to around two hours for hybrid capture. It is now feasible to generate exome-capture data and produce a list of somatic mutations in about three days. Hybrid capture also enhances the sequencing data quality obtained from tumor RNA (cDNA) sequencing, especially for low yield and/or FFPE-derived samples (57).

Detecting somatic mutations. Mutation calling from exome-capture sequencing data is achieved by aligning sequence reads to reference genomes, which serve as the keystone for analyzing the short read lengths (~100 bp) produced by MPS platforms. Once reads are aligned to the genome, variants are identified using several algorithms to interpret different types of mutations, including point mutations (or single nucleotide variants [SNVs]) and focused insertion or deletion variants (indels). Tumor variant calls are then compared with data from a matched normal tissue DNA obtained using a similar capture reagent in order to identify tumor-unique (“somatic”) mutations. Subsequent annotation steps convert variations in nucleic acid sequence to changes in amino acid sequence, thereby providing the initial data required to identify and rank order tumor neoantigens.

One aspect of this process that needs further work is the ability to predict neoantigens arising from more “extreme” mutations. In principle, variants that add or delete an amino acid or truncate or extend open reading frames or fusion genes arising from translocations or inversions could be a source of highly antigenic novel epitopes; however, indel variants have, in the past, been difficult to detect with high certainty, even when algorithms are employed that are specifically tuned to detect this type of mutation. However, recent advances now permit the identification of some indels with a fairly high degree of certainty, although indels containing highly repetitive regions remain a difficulty. Structural variants also are difficult to identify, especially from exome-capture data, and hence are not likely to be easily detected unless RNA sequencing (RNA-Seq) data can be evaluated for fusion transcripts (which has a correlative high false-positive rate). In all cases, the use of RNA data from cDNA capture sequencing (cDNA Cap-Seq) or RNA-Seq to identify and/or confirm such variants is critically important.

Predicting tumor neoepitopes. Currently, the most useful epitope prediction algorithms are those that focus on binding of peptides to MHCI class I (MHCI) molecules. In both humans and mice, the MHCI antigen presentation pathway is responsible for presenting peptides derived from endogenous cell-intrinsic proteins to CD8+ CTL (58). Endogenous proteins are processed by the proteasome (59) and the resulting 8-11 amino acid peptides transported into the ER by the transporter associated with antigen processing (TAP) (60), where they are loaded onto newly synthesized class I molecules and the stabilized peptide–MHCI (p–MHCI) complexes are transported to the cell surface. MHCI alleles are remarkably polymorphic, and the number of potential self and foreign peptides processed by normal, infected, or transformed cells is very large (61). There are nearly 2,500 human MHCI allelic sequences and, because human cells can express as many as six distinct MHCI alleles, the capacity to accurately predict which tumor-derived mutant peptide will bind a particular MHCI is challenging.

Multiple tools exist to predict peptide binding to MHCI. A comprehensive list of prediction tools is available (http://cancerimmunity.org/resources/webtools/), and the bioinformatics and biochemical aspects of these programs have been extensively reviewed elsewhere (62–64). Whereas SYFPEITHI (65), Rankpep (66), and BIMAS (67) were the first such tools to be developed, more accurate prediction algorithms have now come on line, and some have been incorporated into the Immune Epitope Database and Analysis Resource (IEDB) (68). A subset of these algorithms predicts peptide binding to different MHCI variants based on artificial neural networks (ANN), providing predicted IC50 as an output (69). In this category, NetMHC (70) is one of the most commonly used and best validated prediction programs (71, 72). Neural network–based approaches depend on the quality and size of the training set and therefore are likely to be more accurate for the more common alleles. A modified form of NetMHC, NetMHCpan (73, 74), expands the training set by including data from other species, leading to improved accuracy for rare MHC alleles. SMM (75) and SMMPMBEC (76) are examples of a second subset of prediction algorithms that use position-weight matrices to describe statistical preferences from p–MHCI binding data. This approach suppresses noise caused by both experimental error and a limited number of data points present in the training set.

Neoepitope prioritization. For identifying tumor-derived mutant epitopes, most studies use predicted p–MHCI binding affinity as the primary criterion for generating an initial prioritized list of candidate epitopes. Most of the reported studies indicate that natural immune responses to tumor neoantigens are selectively directed to epitopes within a group predicted to have the strongest MHCI binding affinities (44, 77, 78). Peptide/MHCI binding is influenced by two additional parameters — epitope abundance and antigen processing (i.e., protein degradation and peptide transport). Although mass spectrometry could potentially provide information on epitope abundance and is rapidly becoming more sensitive, the current sensitivity and requirement for large numbers of tumor cells remain as important impediments in employing this approach to identifying the entire peptidome expressed on MHCI (79). Therefore, epitope abundance is currently estimated indirectly by quantitating RNA expression levels. In one approach, mutations defined by tumor-to-normal DNA comparisons are subjected to bioinformatic analysis to predict their immunogenicity and the levels of candidate immune stimulatory peptides are estimated by RNA-Seq. RNA evaluation provides information regarding (a) whether the variant is expressed in the RNA and (b) the mutant allele’s expression level relative to other genes. In a second approach, cDNA capture is performed from tumor RNA
and compared with normal DNA to provide a list of mutated peptides. Due to the error rate of reverse transcriptase and sources of false positivity in variant calling in RNA versus DNA, the predicted mutations are refined further by applying a series of filters to remove known sources of false-positive variants (e.g., low coverage in tumor or normal, or low numbers of variant-containing reads) and to eliminate nonexpressed genes and/or alleles. A final filter eliminates genes with low expression that have a fragment per kilobase of million mapped reads (FPKM) of less than one. Downstream steps of immunogenicity prediction can then be made for this filtered set of mutant peptides. Additional algorithms exist to refine epitope predictions including those focused on defining proteasomal cleavage (i.e., NetChop) (80) and/or TAP transport (i.e., NetCTL and NetCTLpan) (81, 82). However, because the latter are currently not as reliable as the MHC-affinity prediction algorithms, they should be used with caution.

In contrast to predicting MHCI epitopes, the accuracy of predicting MHCII epitopes has been problematic. Whereas the MHCI binding groove is closed at both ends, MHCII has a peptide-binding groove that is open, leading to considerable variation in both the length of peptides that can bind to MHCII and the location of the binding core. Multiple MHCII binding prediction algorithms are available (such as TEPITOPE, ref. 83; netMHCII, ref. 84; and SMM-align, ref. 85); however, they have not been extensively used in the past to identify tumor-specific mutant MHCII epitopes.

Recent preclinical studies illustrating the use of personalized TSAs in immunotherapy

MHCI neoepitopes. Two recent preclinical studies demonstrated the feasibility of developing personalized neoantigen-based cancer immunotherapies. Using a combination of mass spectrometry, next generation sequencing, and bioinformatics approaches, Yadav, Lill, Delamarre, and colleagues identified seven mutant neoepitopes bound to MHCI expressed on MC38 mouse colon adenocarcinoma cells (78). Two of the stronger H-2Db−binding epitopes, derived from point mutations of Reps1 and Adpgk, induced antigen-specific CD8+ T cell responses in naive WT syngeneic mice when administered together with polyinosinic:polycytidylic acid (poly I:C) and agonistic anti-CD40. One H-2Kb− epitope, derived from a point mutation of Dpagt1, also induced a weak T cell response. Therapeutic vaccines comprising synthetic long peptides (SLP) containing the three positive epitopes provided mice bearing established MC38 tumors with a significant degree of therapeutic protection.

An independent study published concomitantly by our group took the approach of determining the tumor antigen specificities of CD8+ T cells responsible for rejecting tumors in mice undergoing
checkpoint blockade immunotherapy (77). In this study, we used an edited mouse MCA sarcoma line (T3) that formed progressively growing tumors in naive syngeneic WT mice, which were rejected upon treatment with mAbs specific for two different immune checkpoints — CTLA-4 and programmed death-1 (PD-1) (Figure 1). As assessed by cDNA-Cap-Seq and a recent refinement to calling mutations, T3 expressed approximately 2,200 nonsynonymous point mutations. Putative epitopes were predicted using three MHCI binding algorithms (SMM, NetMHC, and NetMHCpan) and calculating the median binding affinities for each epitope. Additionally, we applied filters for proteasomal processing (NetChop), neoepitopes (i.e., where the mutant epitope bound to MHCI in a manner equal or greater to WT sequence), and deprioritization of hypothetical proteins. Using this approach, two MHCI H-2Kb–restricted tumor neoantigens were identified: point mutations in α,1,3-glucosyltransferase (Alg8) and laminin α subunit 4 (Lama4). These predictions were validated by the following observations. First, primary TILs from T3 tumors were specifically stained by H-2Kb tetramers carrying either ALG8 or LAMA4 (but not other predicted epitopes), when stimulated with irradiated splenocytes and/or TNF-α. Second, the same two mutant epitopes were recognized by pulsing either ALG8 or LAMA4 (but not other predicted epitopes), or buffer (A) or therapeutically treated with mAbs to CTLA-4 and/or PD-1 immune checkpoints (B). Adapted with permission from Nature (ref. 77; Figure 1A and Figure 2, D and E).

Figure 2. Mutant neoantigen–specific peptide vaccines induce therapeutic effects comparable to those of checkpoint blockade therapy. Kaplan-Meier survival curves of tumor-bearing mice therapeutically vaccinated with a vaccine comprising poly I:C plus either ALG8 plus LAMA4 SLP control SLP (HPV peptide), or buffer (A) or therapeutically treated with mAbs to CTLA-4 and/or PD-1 immune checkpoints (B). Adapted with permission from Nature (ref. 77; Figure 1A and Figure 2, D and E).
corresponding normal tissue of similar age was available. Nevertheless, the two studies support the conclusion that T cell–dependent immune control of cancer is dependent on the presence of both mutant MHCII and mutant MHCI tumor neoepitopes.

**Therapeutic use of tumor-specific mutant antigens in human cancer**

In the past year, the first examples of TSA-based personalized cancer immunotherapies have begun to emerge. In 2014, the Rosenberg group used a novel TSA-based personalized adoptive cell therapy (ACT) method to treat a patient with metastatic cholangiocarcinoma (91). Specifically, 26 nonsynonymous mutations were identified in a lung metastasis from the patient by whole-exome sequencing, and candidate minigene constructs for each mutation were transfected into patient-derived APCs. TILs from lung metastases were screened for reactivity with transfected APCs, leading to identification of a point mutant ERBB2-interacting protein as a neoantigen recognized by CD4+ T cells within the TIL population. TILs enriched to 25% for reactivity to the neoantigen were then infused back into the patient, who showed a partial response to the first ACT treatment and showed improved responses to subsequent treatments with TIL preparations enriched to 95% of neoantigen-specific CD4+ T cells. These results not only demonstrated a therapeutic advantage to personalizing ACT therapy, but also specifically documented the therapeutic efficacy of neoantigen-specific CD4+ T cells.

Using more classical vaccine approaches, Carreno et al. recently showed that tumor neoantigens, when administered as a vaccine to three melanoma patients, can enhance both preexisting anti-tumor T cell responses and induce responses to neoepitopes that were undetectable prior to vaccination (92). Thus, these studies support the preclinical work that preceded them and show that vaccines comprising IFN-γ/CD40 ligand–activated dendritic cells pulsed with tumor neoepitopes represent yet another method to deliver personalized cancer vaccines to tumor-bearing individuals (Figure 3).

The idea that the neoantigen landscape might predict clinical responses to checkpoint blockade therapy was proposed in recent work from Snyder, Wolchok, and Chan, where a mutational gene signature was inferred that correlated with long-term clinical benefit of anti–CTLA-4 (ipilimumab) therapy (93). While the mutational load alone was found to partially predict long-term clinical benefit, specific four–amino acid motifs within the NetMHC-predicted neoepitopes were claimed to be even better predictors of clinical response. However, this claim has been recently challenged because other investigators have not been able to find such motifs in validated neoepitopes. This same group together with the Schumacher group subsequently demonstrated that higher mutational burden was also associated with improved responses and progression-free survival in patients with non–small cell lung cancer treated with anti–PD-1 (pembrolizumab) (94). Response was also correlated with mutations in genes encoding components in the DNA-repair pathway that resulted in a higher number of predicted neoantigens. For the one patient in the cohort whose tumor neoantigen was defined, neoantigen-specific T cell responses paralleled tumor regression, implicating a link between the T cell responses and the antitumor effects of anti–PD-1 therapy. A recent report by Chowell et al. used bioinformatics approaches to support an argument that the hydrophobicity of TCR contact residues...
form a hallmark of immunogenic epitopes (95). Thus it is clear that more work is needed to clarify whether there are amino acid motifs that play important roles in determining neoantigenicity.

Concluding remarks
The last decade has seen an explosive growth in our appreciation of the role of immunity in cancer. We now know that the immune system recognizes, shapes, and/or promotes cancer development or outgrowth (22, 41–43, 96, 97). Based on our enhanced insight into immune system/cancer interactions, new immunologic approaches to treating cancer have been developed, often leading to durable clinical responses in a subset of cancer patients (47, 98–100).

Two particular characteristics have brought cancer immunotherapy to the forefront of modern molecular medicine (101). First, this therapy can be staged such that it induces complete responses or stable disease with less collateral damage than conventional chemotherapy or radiation therapy. Second, because the immune response can continuously evolve new specificities, cancer immunotherapy has the capacity to respond reactively to genetically unstable, continuously changing cancer cell target populations. However, current cancer immunotherapies still display inherent toxicities because they function within the narrow therapeutic space between tumor immunity and autoimmunity and induce responses only in a subset of patients. The use of TSAs as the basis for personalized cancer immunotherapy offers the potential to make the therapy more specific, more effective, and safer compared with the cancer immunotherapies that we have available today. However, key questions remain to be answered. Although we have achieved a level of competency in identifying point mutations and indels that can form MHCI epitopes, there remain several other potential tumor neoantigens (such as products of gene fusions, proteins arising from mistakes in translation, or mutant proteins that form MHCII antigens) that are currently not easy to predict with bioinformatics tools. We need to resolve the important questions of whether tumor-specific mutant antigens (or the closely related group of shared driver neoantigens that could reduce the chances of escape variant formation) really offer unique opportunities to target tumor cells compared with shared, nonmutated TAAs. Furthermore, we need to determine what minimum number of neoepitopes must be employed to reduce the likelihood of formation of antigen-loss variants. Finally, we need to experimentally establish that therapeutically targeting tumor neoantigens does not lead to unanticipated autoimmune consequences. Even after we resolve these issues, we still need to determine how best to use this information therapeutically. Is it through defining the best types of personalized cancer vaccines, individualized adoptive cell therapies, some combination of these together with checkpoint blockade immunotherapy, or even combinations of immunologic and standard cancer immunotherapies? Strategies targeting different aspects of the “cancer-immunity cycle” (102) (i.e., priming and/or effector immunity) with checkpoint blockade may be helpful in contextualizing the role of targeting TSAs within the multistep process of effective antitumor immunity. Will the methods that are ultimately used need to be employed in a manner that is based on the type of cancer being treated? We envisage that these types of questions will be answered in the next decade of cancer immunology, genomics, and immunotherapy research. It is clear that we now stand at an important crossroads of opportunities to enhance our ability to use the immune system to make cancer a controllable and, in some cases, curable disease. We predict that the capacity to personalize cancer immunotherapy will contribute significantly to this important endeavor.

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