Immunopharmacogenomics towards personalized cancer immunotherapy targeting neoantigens

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Utilizing the host immune system to eradicate cancer cells has been the most investigated subject in the cancer research field in recent years. However, most of the studies have focused on highly variable responses from immunotherapies such as immune checkpoint inhibitors, from which the majority of patients experienced no or minimum clinical benefit. Advances in genomic sequencing technologies have improved our understanding of immunopharmacogenomics and allowed us to identify novel cancer-specific immune targets. Highly tumor-specific antigens, neoantigens, are generated by somatic mutations that are not present in normal cells. It is plausible that by targeting antigens with high tumor-specificity, such as neoantigens, the likelihood of toxic effects is very limited. However, understanding the interaction between neoantigens and the host immune system remains a significant challenge.

This review focuses on the potential use of neoantigen-targeted immunotherapies in cancer treatment and the recent progress of different strategies in predicting, identifying, and validating neoantigens. Successful identification of highly tumor-specific antigens accelerates the development of personalized immunotherapy with no or minimum adverse effects and with a broader coverage of patients.

KEYWORDS
cancer precision medicine, immune checkpoint inhibitor, neoantigen, next-generation sequencing, T-cell receptor repertoire

1 | INTRODUCTION

Due to the development of immunotherapies, in particular, immune checkpoint inhibitors as well as recent advances in the technology to sequence cancer genomes and to characterize T-cell or B-cell receptors, immunogenomics or immunopharmacogenomics have received a very high level of attention in the cancer research field. Tumor-infiltrating T lymphocytes, which have been shown to associate with better prognosis in patients with various solid cancers,1,2 are recognized as an important key factor in cancer immunotherapy. They exert their adaptive immune responses through the recognition of antigens that are specifically expressed on cancer cells. However, the mechanism that allows the immune system to distinguish between benign cells and tumor cells is still not well understood. Recent research has confirmed that neoantigens, which are generated by somatic genetic mutations in cancer cells, can be recognized as “non-self” antigens by the immune system. Such a unique characteristic highlights the important roles of neoantigens and their potential contribution in immunotherapy.

2 | IMMUNE CHECKPOINT INHIBITORS

Immunotherapies such as immune checkpoint antibodies have revolutionized cancer treatments. Immune checkpoint antibodies target the inhibitory molecules that suppress the host immune response
against cancer cells and then allow the activation of the host immune system, resulting in eradication of cancer cells. The major targets for such blockade in the clinic are cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), and the interaction between programmed death-1 (PD-1) and PD-1 ligand-1 (PD-L1). Both CTLA-4 and PD-1/PD-L1 are key regulators that are responsible for suppression of T-cell-mediated antitumor immune responses. However, recent meta-analysis of clinical data clearly showed that only a subset of patients responded to immune checkpoint inhibitors; the majority of patients had no or minimum clinical benefit and some suffered from severe immune-related adverse reactions. Such observation highlights the importance of identifying a predictive biomarker(s) that can be used to select patients who are more likely to expect clinical benefit with minimal risk of autoimmune adverse events, contributing to reduction of unnecessary medical costs.

High PD-L1 expression in cancer cells and high infiltration of immune cells have been suggested to correlate with clinical responses to anti-PD-1 therapy. Contrarily, it has been shown in several studies that patients who lacked the PD-L1 expression in cancer tissues showed clinical benefit from anti-PD-1 therapy. Another PD-1 ligand, PD-L2, is also known to suppress cytotoxic activity of T cells in tumors; thereby high PD-L2 expression could be associated with treatment response. Several studies have investigated predictive biomarkers for response to immune checkpoint antibodies. We previously reported that the expression levels of granzyme A and human leukocyte antigen (HLA)-A in melanoma tissues before treated with anti-PD-1 antibody were significantly higher in responders compared to non-responders, which could indicate their value as potential biomarkers to predict clinical responses. Recent reports suggest that loss of heterozygosity of HLA class I locus is an immune escape mechanism that is related with poor outcome in patients who received immune checkpoint inhibitor therapy. Furthermore, we showed that the T-cell receptor (TCR) repertoire of tumor-infiltrated T cells might be useful information for the evaluation or assessment of the immune microenvironment. Recent reports, including ours, suggested that durable response to immune checkpoint blockade correlated with early and sustained expansion of a few dominant T-cell clones in peripheral blood, and that TCR sequencing can be used to detect early signs of response/resistance to immune checkpoint blockade.

A series of studies have also addressed the relationship between mutational burden and immunotherapeutic outcome. Early studies supported the hypothesis that the extent of DNA damage is correlated with therapeutic response in melanoma patients treated with anti-CTLA-4 antibody, and non-small-cell lung cancer patients treated with anti-PD1 antibody. A more recent study also found the predictive power for neoantigen load in anti-CTLA-4-treated melanoma patients. More importantly, mismatch-repair proficiency was associated with clinical response; it was observed that patients who have a higher number of somatic mutations due to the mismatch-repair deficiency were more susceptible to PD-1 blockade therapy compared to patients without the mismatch-repair deficiency. The results from these studies all indicated a strong positive relationship between the numbers of predicted neoantigens, host immunological responses, and overall survival. A high somatic mutation burden should theoretically increase the probability of generating neoantigens. These tumor antigens can then be presented with HLA molecules to the surface of cancer cells and be recognized by CD8+ T cells to enhance antitumor immune responses.

### 3 | CANCER-SPECIFIC ANTIGENS

The general mechanism of how the host immune system attacks the cancer cells has been well studied. It is believed that as the T cells infiltrate into tumors, they recognize the HLA-bound cancer-specific peptides on the cancer cell surface, are activated and proliferate, and kill the cancer cells. The tumor antigens are generally classified into several types: (i) overexpressed antigens that are more highly expressed in cancer cells compared to normal cells (e.g., melanoma-associated antigen recognized by T cells [MART-1] and glycoprotein 100 [gp100]); and (ii) tumor-specific and shared antigens that are expressed in cancer and/or testis, but not in other normal tissues (e.g., melanoma-associated antigen 1 [MAGE-A1], NY-ESO-1, upregulated gene in lung cancer 10, ring finger protein 43, and kinesin family member 20). These tumor-specific antigens are also called cancer-testis antigens because of their expression patterns. However, some of these antigens have also been found in other normal tissues with low expression. In addition, a new group of antigens, neoantigens, have attracted an attention extensively in recent years due to their high specificity to cancer cells. They are generated by somatic mutations in cancer cells and are not present in normal cells, which therefore can be recognized as “non-self” antigens by the immune system. This concept was first examined during the late 1980s by several groups using mouse models. Recent advances in next-generation sequencers enable us to comprehensively study the genomic landscape of tumors and analyze the tumor microenvironment.

An increasing number of studies has reported the associations of mutational burden/predicted neoantigen load and the intratumoral immune infiltrates with patient survival. Rooney et al. analyzed whole-exome sequencing and transcriptome datasets from thousands of solid tumors across 18 tumor types in The Cancer Genome Atlas (TCGA). They identified that cytolytic activity of CTLs, estimated from the expression levels of key immune effectors, granzyme A and perforin 1, was correlated with somatic mutation load and the numbers of predicted neoantigens. Brown et al. also analyzed RNA sequencing data for 515 patients from 6 tumor sites from TCGA dataset and reported similar results, in which higher predicted neoantigen load was significantly associated with better prognosis. Interestingly, these studies found that tumors with 150-200 nonsynonymous somatic mutations showed an elevated cytolytic T cell score. Several other studies supported that higher predicted neoantigen load was also associated with lymphocytic infiltration into tumors and survival rate. These associations were observed across both microsatellite stable and unstable tumors in colorectal and ovarian cancer patients. In 2014, we also reported similar findings on the association between somatic mutations and survival rate.
using whole-exome and target sequencing in 78 muscle-invasive bladder cancer patients. We found that carriers of somatic mutations in DNA repair genes had significantly better recurrence-free survival compared with non-carriers (median, 32.4 vs 14.8 months; hazard ratio, 0.46; \( P = 0.044 \)). The follow-up study identified that tumors with higher neoantigen load showed lower TCR diversity, which correlated with oligoclonal tumor-infiltrating T lymphocyte expansion, and showed a significant association with longer recurrence-free survival (hazard ratio, 0.37; \( P = 0.033 \)). We also described the correlation between higher neoantigen load and T-cell activation among different portions within the same tumors.

4 | NEOANTIGEN PREDICTION

It is still a major challenge to accurately predict the interaction between neoantigens and immune cells. There are currently several publicly available neoantigen prediction pipelines, including pVACSeq,

\[ \text{INTEGRATE-neo}, \text{TSNAD} \]

pVAC-Seq combines the tumor mutation and expression data to predict neoantigens by invoking NetMHC 3.4; INTEGRATE-neo was designed to predict neoantigens from fusion genes based on the pipeline INTEGRATE and NetMHC 4.0. Similar to these pipelines, TSNAD also uses widely approved software NetMHCpan 2.8 to predict neoantigens. We have also developed a pipeline to predict neoantigens from whole-exome and RNA sequencing data (Figure 1).

4.1 | Human leukocyte antigen typing based on whole-exome data

In-depth understanding of HLA molecules is vital for accurate neoantigen prediction. The HLA class I molecules are critical mediators of the cytotoxic T-cell responses as they present antigen peptides on the cell surface to be recognized by TCR on T cells. The HLA gene cluster, located on the short arm of chromosome 6, is among the most polymorphic regions in the human genome, with thousands of documented alleles. The HLA allele has a unique nomenclature that comprises the gene name indicating the locus (i.e., A, B, or C) followed by successive sets of digits separated by colons. The first two digits (field 1) specify the allele groups by serological activity (allele level resolution, ex. A*01 or A*02), and the second field indicates the protein sequence (protein level resolution, ex. A*02:01 or A*02:02). The remaining two sets distinguish synonymous polymorphisms and non-coding variations. At least 2-field HLA typing is required to accurately predict neoepitopes, which can bind to HLA molecules. Several tools have been developed to obtain HLA allele information from genome-wide sequencing data (whole-exome, whole-genome, and RNA sequencing data), including OptiType, Polysolver, PHLAT, HLAreporter, HLAforest, HLAminer, and seq2HLA. We have tested 961 whole-exome data from the 1000 Genomes Project to evaluate the accuracy of these programs. Among these algorithms, OptiType showed the highest accuracy of 97.2% for HLA class I alleles at the second field level, followed by 94.0% in Polysolver and 85.6% in PHLAT.

4.2 | Variant call and RNA expression

Somatic mutation calling from whole-exome sequencing data is achieved by aligning sequence reads to the reference genome, comparing tumor DNAs with matched normal control DNAs to identify single nucleotide variants and insertions/deletions (indels). Although our neoantigen prediction pipeline accepts output from any variant callers, we used the Genomon Exome pipeline to obtain somatic variant information (http://genomon.readthedocs.io/ja/latest/) and

![FIGURE 1](image-url)
extract non-synonymous mutations and indels to translate them into amino acid sequences. We then use RNA sequencing data of tumors to examine gene expression and predict whether each neoepitope could bind to HLA molecules. Most of the tools use expression filtering based on fragments per kilobase of exon per million reads mapped FPKM or reads per kilobase of exon per million reads mapped RPKM, although they cannot distinguish between WT and mutated RNAs. In our pipeline, we created a script to obtain each of the WT RNA expression and mutated RNA expression separately in addition to the total RNA expression reads. The expression of approximately 45.0% (range, 11.1%-72.7%) of mutated genes were detected in RNA sequencing reads. It is currently still disputable how many levels of RNA or protein expression are required for CTLs to detect peptide–HLA complexes and be effectively activated. Although no empirical studies have attempted to address this issue in human trials, it appears that the very low expression level (i.e., even a single peptide–HLA protein/cell) seemed to be sufficient for activation of CTLs.41

4.3 | Prediction of binding affinity to HLA molecules

Genetic variants must result in modified peptides that can be presented on HLA molecules in order to successfully elicit T-cell responses. Different computational methods have been developed to predict peptide binding-affinity to HLA molecules. The HLA class I molecules usually present 8-11 amino-acid peptides to cytotoxic CD8+ T-lymphocytes where the N- and C-termini are involved in HLA binding.42 Therefore, known HLA ligands of equal length can be served as predictive models for the binding affinity. Specific sequence motifs for the HLA binding can be identified by aligning to these known ligands using software including BIMAS. More accurate prediction approaches are based on artificial neural networks with predicted IC50. An example of such an approach is NetMHC.43,44 which is one of the most commonly used and best validated prediction programs. These algorithms are trained using large datasets of peptide ligands such as those collected in the IEDB database.45 therefore, their prediction accuracy is higher for common HLA alleles than with many known ligands. A modified version of NetMHC, NetMHCpan, has been developed to predict peptides binding to alleles for which no ligands have been reported.46 Most algorithms are developed based on the prediction of the affinity of individual peptides to each HLA molecule. However, the affinity to the HLA molecules may not accurately represent the immunogenicity in individual patients, and it has been reported that peptide–HLA complex stability might have a better correlation with the immunogenicity of the peptides.47 Some computational methods, such as NetChop and NetCTL, focus on predicting antigen processing and peptide transport processes.48,49 Structural-based approaches are also important to predict the orientation of a mutated amino acid that is facing out of the HLA molecules to predict unnecessary cross-reaction to a WT peptide. However, the individual parameter mentioned above has limited predictive values for immunogenicity in individual patients and a combination of these factors to predict neoantigens still remains as a big challenge to be addressed. Future developments may benefit from artificial intelligence or machine learning approaches with high-throughput validation and larger datasets of cancer-specific HLA ligands and T cell epitopes.

4.4 | Experimental validation of neoantigen antigenicity

A large diversity of bioinformatics and biochemical tools are available to in silico predict, filter, or experimentally validate candidate peptides on the basis of their processing, HLA binding affinity, and stability. However, experimental validation of the immunogenicity of predicted potential neoantigens using patient’s own T cells still remains as the final confirmation of their potential relevance. Recently, several methodologies were developed to interrogate patient’s cellular immunity. These T cell-based functional assays use either in silico predicted short peptides or tandem minigenes.50 As summarized in Table 1, there are several reports in which neoantigen-specific T cells were successfully induced. We also developed a rapid pipeline to identify TCRs recognizing neoantigens.51 The process takes only approximately 2 weeks. It starts with peptide stimulation and ends with the identification of a TCRβ pair(s), and established TCR-engineered T cells with antigen-specific cytotoxic activity. In 2016, a study found that mutation-reactive T cells could be enriched from donor-derived T cells and used as an effective therapy for the treatment of patients with metastatic cancer.52 However, only 1 in 5-20 predicted neoantigens seems to activate antigen-specific CTLs in cancer patients.

4.5 | Immunopeptidome analysis

Due to the complexity of neoantigen prediction, often only a small fraction of the predicted neoantigens can be confirmed to be associated with neoantigen-driven immune responses. One of the approaches to further narrow down the selected candidates is to assess whether the predicted peptides in fact bind to the HLA molecules using liquid chromatography and tandem mass spectrometry (LC-MS/MS) analysis.53 The best-established strategy to isolate peptides on the HLA is immunoaffinity purification of HLA–peptide complexes using pan-anti-HLA class I or class II antibodies. Typically, 106 to 107 cells are required for in-depth immunopeptidome analysis, which will allow the identification of thousands of peptides presented on HLA molecules.54 Extremely high levels of sensitivity and accuracy of LC-MS/MS analysis are required for detailed analysis of immunopeptidomes. The obtained MS/MS spectra will be compared to theoretical spectra of peptide sequences in databases using search engines like Mascot55 or MaxQuant56 to identify the HLA-binding peptides. As inclusion of known mutations from repositories such TCGA or the COSMIC database may increase false positives, better personalized and customized reference databases based on whole-exome and transcriptome sequencing information allow identification of private peptides that are not present in reference.
protein sequence databases. Unfortunately, although bioinformatic approaches and in-depth analysis enable accurate identification of thousands of HLA peptides, only a few neoepitopes derived from predicted neoantigens have been identified. These results suggest a possible underdetection of neoantigens with post-translational modifications or low levels of neoantigen expressions.57,58 The HLA class II immunopeptidome has also been previously examined.54,59,60 Immunopeptidome analysis has a big advantage in identifying highly expressed neoepitopes on HLA molecules in cancer cells; however, enhanced assay sensitivity with improved bioinformatics analysis are critically essential to maximize the potential of immunopeptidome analysis for clinical application. It should be noted that functional assays will be definitely required to accompany immunopeptidome analyses in order to assess the immunogenicity of the identified neoantigens.

### 5 NEOANTIGEN-BASED PERSONALIZED IMMUNOTHERAPY

Several approaches have been extensively studied in recent years to enhance CTL-mediated antitumor immune responses in cancer immunotherapies (Figure 2). Cancer-peptide vaccines targeting cancer-specific neoantigens and shared antigens can activate or induce antigen-specific CTLs in cancer patients. The immunogenicity of shared antigens has been established in many clinical studies, including ones reported by our group.61-63 As previously mentioned, neoantigens are immunogenic non-self-peptides that are generated by somatic mutations in cancer cells; therefore, they have attracted much attention as cancer cell-specific antigens.64 Neoantigen-specific T cells have already shown positive clinical results.65,66 Recently, the results of 2 phase I clinical trials were reported that assessed a personalized neoantigen vaccine approach to treat patients with melanoma.67,68 Both studies found that the vaccine treatment induced neoantigen-specific CD4+ and CD8+ T cells, and indicated potential benefits of neoantigen-based vaccines. Ott et al. showed that 4 of 6 vaccinated patients had no recurrence at 25 months after the vaccination, and 2 patients with recurrent disease had complete regression by subsequent treatment with anti-PD-1 antibodies. Sahin et al. showed that 8 of 13 vaccinated patients mounted strong immune responses and remained recurrence-free for 12-23 months. Two of the remaining 5 patients with metastatic disease experienced vaccine-related objective responses. These results indicate that neoantigen vaccines can be a promising treatment option to induce neoantigen-specific T cells in cancer patients.

#### TABLE 1 Summary of studies identifying neoantigen-specific T cells

| Study | Type of cancer | Source | Methods | No. of patients | No. of tested (predicted) peptides | No. of detected T-cell responses |
|-------|----------------|--------|---------|-----------------|-----------------------------------|---------------------------------|
| van Rooij et al., 2013 | Melanoma | TILs | pHLA multimer | 1 | (448) | 1 |
| Robbins et al., 2013 | Melanoma | TILs | ELISPOT | 3 | 227 (247) | 11 |
| Wick et al., 2014 | Ovarian cancer | TALs | ELISPOT | 2 | 114 | 1 |
| Rajasagi et al., 2014 | CLL | Patients’ PBMCs | ELISPOT | 2 | 48 | 3 |
| Lu et al., 2014 | Melanoma | TILs | ELISA | 2 | 288 | 2 |
| Snyder et al., 2014 | Melanoma | Patients’ PBMCs | Intracellular cytokine staining | 2 | – | 2 |
| Rizvi et al., 2015 | NSCLC | Patients’ PBMCs | pHLA multimer | 1 | (226) | 1 |
| Cohen et al., 2015 | Melanoma | TILs | pHLA multimer | 8 | 459 | 9 |
| Linnemann et al., 2015 | Melanoma | TILs | ELISA | 3 | 460 | 4 |
| Carreno et al., 2015 | Melanoma | Patients’ PBMCs | pHLA multimer | 3 | 21 | 6 |
| Kalaora et al., 2016 | Melanoma | TILs | pHLA multimer | 1 | 2 | 1 |
| McGranahan et al., 2016 | NSCLC | TILs | pHLA multimer | 2 | 642 | 3 |
| Stronen et al., 2016 | Melanoma | Donors’ PBMCs | pHLA multimer | 4 | 56 | 11 |
| Bassani-Stemberg et al., 2016 | Melanoma | TILs | ELISPOT/pHLA multimer | 1 | 8 | 2 |
| Gros et al., 2016 | Melanoma | Patients’ PBMCs | ELISPOT | 4 | 691 | 7 |
| Bentzen et al., 2016 | NSCLC | TILs | pHLA multimer | 2 | 703 | 9 |
| Tran et al., 2016 | Gastrointestinal cancer | TILs | ELISPOT | 9 | 1273 | 18 |
| Kato et al., 2016 | Cell line, ovarian cancer | Donors’ PBMCs | pHLA multimer | 2 | 17 | 2 |

–, not available; CLL, chronic lymphocytic leukemia; ELISPOT, enzyme-linked immunospot assay; NSCLC, non-small-cell lung cancer; pHLA, peptide–human leukocyte antigen; TAL, tumor-associated lymphocyte; TIL, tumor-infiltrating lymphocyte.
Furthermore, adoptive immunotherapies using TCR-engineered T cells are being extensively studied as they have shown promising results in recent clinical trials. In this approach, identification of cancer antigen-specific TCR and infusion therapy with TCR-engineered T cells can induce very effective CTL-mediated anticancer immunity, particularly for advanced tumors where the host immune system was extensively suppressed. The first adoptive T-cell therapy with TCR-engineered T cells was reported in 2006. The MART-1 TCR-modified lymphocytes successfully mediated tumor regression in humans. The NY-ESO-1-specific TCR-engineered T cells are the most thoroughly examined, and objective responses were observed in more than 50% of patients with synovial cell carcinoma, melanoma, and myeloma. Currently, there is no published data on neoantigen-specific TCR-engineered T-cell therapy in humans. However, preclinical studies have shown encouraging results where neoantigen-specific TCR-engineered T cells are also effective for large-sized solid tumors.

6 | CONCLUSION

Based on the results from research in recent years, it is plausible that neoantigens are a promising therapeutic target for T-cell cancer immunotherapies. The tumor-restricted expression of neoantigens highlights their specificity and potential safety in clinical settings. However, the key rate-limiting factor for the progress of neoantigen-specific T-cell therapy is the insufficient accuracy of neoantigen prediction, including the HLA-binding affinity, the immunogenicity of predicted peptides in individual cancer patients, and also the cross-reactivity to WT peptides. Further studies are required to establish precise neoantigen prediction with efficient validity assays. In addition, accurate prediction in selecting the patient groups that may benefit from neoantigen-targeted immunotherapies will further reduce the likelihood of unwanted adverse effects.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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