Neoantigens in precision cancer immunotherapy: from identification to clinical applications

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Abstract

Immunotherapies targeting cancer neoantigens are safe, effective, and precise. Neoantigens can be identified mainly by genomic techniques such as next-generation sequencing and high-throughput single-cell sequencing; proteomic techniques such as mass spectrometry; and bioinformatics tools based on high-throughput sequencing data, mass spectrometry data, and biological databases. Neoantigen-related therapies are widely used in clinical practice and include neoantigen vaccines, neoantigen-specific CD8+ and CD4+ T cells, and neoantigen-pulsed dendritic cells. In addition, neoantigens can be used as biomarkers to assess immunotherapy response, resistance, and prognosis. Therapies based on neoantigens are an important and promising branch of cancer immunotherapy. Unremitting efforts are needed to unravel the comprehensive role of neoantigens in anti-tumor immunity and to extend their clinical application. This review aimed to summarize the progress in neoantigen research and to discuss its opportunities and challenges in precision cancer immunotherapy.

Keywords: Precision cancer immunotherapy; Neoantigen; Anti-tumor immunity; T cells; Vaccination

Introduction

Antitumor treatment has entered a new era since the successful application of immunotherapies, of which a growing number have emerged. Immune checkpoint inhibitors (ICIs), adoptive transfer of T/natural killer (NK) cells, tumor-associated antigen (TAA) vaccines, oncolytic viruses, and immunomodulators have been proven to be effective in clinical practice.[1-4] However, even ICIs, the most popular immunotherapeutic agents, are only permitted to treat a few specific types of cancer. Results from a large number of clinical trials show that the objective response rates (ORRs) of most cancer immunotherapies are far from satisfying, let alone their association with frequent immune-related adverse events (irAEs).[5,6] Therefore, cancer immunotherapy must be more precise to achieve higher ORRs and fewer irAEs. Facing this challenge, pioneers have made considerable efforts to discover appropriate targets. Among the targets discovered, neoantigens are ideal for precision cancer immunotherapy.[7]

Neoantigens are abnormal peptides specifically expressed by malignant cells that are present on their surfaces. Most neoantigens are products of accumulating mutations in somatic cells. In virus-associated cancers, such as cervical cancer, neoantigens may be the products of the open reading frames of viral genomes.[8] In contrast to TAAs, neoantigens can activate $CD4^+$ and $CD8^+$ T cells exempt from central tolerance because they are totally non-self for the immune system.[9] Identification of neoantigens by researchers can be traced back to the 1980s.[10] Initially, complementary DNA library screening was used to identify neoantigens.[11] With the application of next-generation sequencing (NGS) technology and bioinformatic algorithms, the prediction of neoantigens became cheaper, easier, and faster.[12] However, prediction results are sometimes unsatisfactory because of the false-positive rate. By combining NGS with mass spectrometry and bioinformatics tools, the identification of neoantigens becomes more accurate. Thus, precision cancer immunotherapy targeting neoantigens has become more feasible.

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Immunotherapies targeting neoantigens are safe, precise, and have demonstrated their potential in numerous clinical trials. Personalized neoantigen vaccines have proven to be feasible, safe, and effective.[9] Adoptive cell therapy targeting neoantigens has also achieved encouraging results.[13] Neoantigens are also important biomarkers for immunotherapy. Taking all these points into consideration, we think it is quite significant to make a comprehensive review of neoantigens and to discuss their future roles in immunotherapy.

Immune Responses Induced by Neoantigens

Immunogenicity of neoantigens

In 1988, researchers discovered that neoantigens could promote the proliferation of cytotoxic T cells in a mouse tumor model.[11] Eight years later, another group of researchers discovered that neoantigens could be recognized by tumor-infiltrating lymphocytes (TILs) in a patient with melanoma.[14] An increasing number of studies have verified the immunogenicity of neoantigens. Briefly, immunogenicity is the ability of neoantigens to activate adaptive immune responses. Neoantigens are treated as non-self by the immune system because they are abnormal peptides expressed by mutated genes in cancer cells. Without the limitation of central tolerance, neoantigens can induce immune responses, as has been proven [Table 1].

However, because of the genomic instability of cancer cells, the heterogeneity of neoantigens is so significant that their immunogenicity varies. The structure of the neoantigen is not the only factor that affects its immunogenicity. The way it is processed and presented is also an important factor. After release from cancer cells, neoantigens are captured by antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages. Neoantigens are degraded into small peptides by the proteasome and then loaded into major histocompatibility complex (MHC) molecules and presented on the surface of APCs.[15] Then, neoantigen-loaded APCs migrate into tumor-draining lymph nodes. When the relative location of APCs and T cells is convenient for interaction, specific T cells recognize neoantigen-MHC complexes by T cell receptors (TCRs). Hence, the spatial location of APCs is another critical factor.

T cell responses

The successful presentation of highly immunogenic neoantigens does not necessarily induce an effective T cell response. Multiple negative co-stimulatory molecules and mechanisms impair T cell activation. For example, immune checkpoints are negative mechanisms that inhibit T cell activity. Many clinical trials focusing on ICIs have proved that immune checkpoint blockade (ICB) is an effective strategy to restore the activity of T cells. ICB has been shown to enhance the durable neoantigen-specific T-cell responses.[16] Perumal *et al*[17] found that the activation and expansion of neoantigen-specific T-cells are improved after treatment with ICIs. Their work showed that therapy based on neoantigens can induce stronger T cell responses in combination with ICB.

In addition, there are many positive co-stimulators of T cell activity. Both interleukin (IL)-2 and IL-15 stimulate the activation and proliferation of $CD8^+$ T cells and NK cells. Although numerous clinical studies have proven that neoantigen vaccines or adoptive neoantigen-specific T cells can achieve satisfactory anti-tumor immunity alone [Figure 1], combination with positive co-stimulators can improve anti-tumor immunity.[18,19] In addition, the IL-2/CD25 fusion protein has been shown to amplify neoantigen-specific CD4+ and CD8+ T cell responses.[20] In contrast to IL-2 alone, the IL-2/CD25 fusion protein is more selective, effective, and less toxic. The implication of this study is that the traditional positive co-stimulators, which may have severe side effects, can be engineered into safer ones. This will accelerate their application in immunotherapy, especially therapies based on neoantigens.

Memory T cells

Neoantigens can induce effector T cells and memory T cells. After exposure to neoantigens, specific T cells mature from naïve cells to effector cells and then eventually to exhausted cells or memory cells. This is the foundation of long-term remission in patients with cancer after immunotherapy based on neoantigens. The number of memory cells among TILs correlates with the outcomes of patients receiving immunotherapy.[21] Although CD8+ T cells are considered the most common subtype of memory T cells,[22,23] Hu *et al*[24] showed that neoantigen vaccines can induce $CD4^+$ memory T cells that persist for years in patients with melanoma. Further analysis of long-term TCR sequencing data and flow cytometry data showed that the clonal memory T cells were $CD4^+$ $\alpha\beta$T cells. A recent study showed that memory T cells can recognize specific antigens as well as shared ones.[25] This finding suggests that personalized neoantigen vaccines can be applied to multiple patients with the same somatic mutation.

Neoantigen Identification

During the past several years, a number of analytical pipelines for neoantigen discovery have been proposed to predict peptides that have the potential to induce tumor immune responses related to T-cell activation.[26-28] Binding affinity between the peptides and MHC is successfully used to select neoantigens against colorectal tumor[29], melanoma[9] and glioblastoma.[30,31] Fitness model integrating binding affinity between MHC and peptide with sequence similarity of peptide and virus sequence is used to identify neoantigens in melanoma, lung and pancreatic cancer.[32,33] Differential agretopicity index model evaluating the binding affinity difference with MHC between wild-type peptide and mutant peptide is applied upon melanoma and lung cancer.[34] High quality neoantigen model is successfully developed to determine neoantigens for primary isocitrate dehydrogenase wild-type glioblastoma.[35] NeoScreen, a recently developed *in vitro* TIL expansion and screening methodology, is reported to enable the selective expansion of neoantigen-targeting TILs against melanoma, colon, lung and ovarian cancers.[36] NeoScreen first applies computational

Table 1: Clinical trials on cancer neoantigen vaccine, retrieved from clinicaltrials.gov.

NCT Number	Patients	Status	Phases	Number of enrolled patients	Arms and interventions	Results
NCT04749641	Diffuse intrinsic pontine glioma	Recruiting	Phase 1	30	Group A: A total of 15 subjects with open surgical biopsy indications will receive microsurgical resection, followed by conformal radiotherapy and administration of the researched vaccine; Group B: A total of 15 subjects without open surgical biopsy indications will receive stereotactic biopsy, followed by conformal radiotherapy and administration of the researched vaccine	
NCT03929029	Melanoma	Recruiting	Phase 1	20	Single arm: nivolumab + ipilimumab + NeoVax plus montanide	
NCT04810910	Resectable pancreatic cancer	Recruiting	Phase 1	20	Single arm: personalized neoantigen vaccines	
NCT03715985	Malignant melanoma, metastatic; non-small cell lung cancer, metastatic; bladder urothelial carcinoma, metastatic	Active, not recruiting	Phase 1, Phase 2	12	Single arm: NeoPepVac	
NCT03558945	Pancreatic tumor	Recruiting	Phase 1	60	Group A: personalized neoantigen vaccine; Group B: conventional treatment	
NCT05192460	Gastric cancer; esophageal cancer; liver cancer	Recruiting	Not applicable	36	Single arm: neoantigen tumor vaccine and PD-1/L1	
NCT03359239	Urothelial/bladder cancer	Completed	Phase 1	10	Single arm: PGV 001 with Atezolizumab	
NCT04487093	Non-small cell lung cancer	Recruiting	Phase 1	20	Group A: neoantigen vaccine + EGFR-TKI; Group B: neoantigen vaccine + anti-angiogenesis drug	
NCT04251117	Hepatocellular carcinoma	Recruiting	Phase 1, Phase 2	24	Single arm: GNOS-PV02 + INO-9012 + pembrolizumab	
NCT03606967	Anatomic stage IV breast cancer; AJCC v8 invasive breast	Recruiting	Phase 2	70	Single arm: neoantigen vaccine, durvalumab, nab-paclitaxel	

Table 1
(continued).

NCT Number	Patients	Status	Phases	Number of enrolled patients	Arms and interventions	Results
	carcinoma; metastatic triple-negative breast carcinoma; prognostic stage IV breast cancer AJCC v8					
NCT04248569	Fibrolamellar hepatocellular carcinoma	Recruiting	Phase 1	12	Single arm: DNAJB1-PRKACA peptide vaccine, nivolumab, and ipilimumab	
NCT04117087	Colorectal cancer; pancreatic cancer	Recruiting	Phase 1	30	Single arm: KRAS peptide vaccine, nivolumab, and ipilimumab	
NCT03794128	Non-small cell lung cancer; colorectal cancer; gastroesophageal adenocarcinoma; urothelial carcinoma; pancreatic ductal adenocarcinoma	Completed	Not Applicable	93	Group A: patient-specific neoantigen cancer vaccine production; Group B: shared neoantigen cancer vaccine screening	
NCT03645148	Pancreatic cancer	Completed	Phase 1	7	Single arm: iNeo-Vac-P01	
NCT03662815	Advanced malignant solid tumor	Active, not recruiting	Phase 1	30	Single arm: iNeo-Vac-P01	
NCT03953235	Non-small cell lung cancer; colorectal cancer; pancreatic cancer; solid tumor; shared neoantigen-positive solid tumors	Recruiting	Phase 1, Phase 2	144	Single arm: GRT-C903, GRT-R904, nivolumab, ipilimumab	
NCT01970358	Melanoma	Completed	Phase 1	20	Single arm: Personalized Neoantigen cancer vaccine	
NCT03639714	Non-small cell lung cancer; colorectal cancer; gastroesophageal adenocarcinoma; urothelial carcinoma	Active, not recruiting	Phase 1, Phase 2	214	Single arm: GRT-C901, GRT-R902, nivolumab, ipilimumab	
NCT03300843	Melanoma; gastrointestinal cancer; breast cancer; ovarian cancer; pancreatic cancer	Terminated	Phase 2	1	Single arm: peptide loaded dendritic cell vaccine	The participant had adverse events like nausea, fatigue, injection site reaction, headache

Table 1
(continued).

NCT Number	Patients	Status	Phases	Number of enrolled patients	Arms and interventions	Results
						and skin induration.
NCT04912765	Hepatocellular carcinoma; colorectal carcinoma	Recruiting	Phase 2	60	Single arm: neoantigen dendritic cell vaccine and nivolumab	
NCT04364230	Melanoma	Recruiting	Phase 1, Phase 2	44	Single arm: 6MHP, NeoAg-mBRAF, polyICLC, CDX-1140	
NCT03480152	Melanoma; colon cancer; gastrointestinal cancer; genitourinary cancer; hepatocellular cancer	Terminated	Phase 1, Phase 2	5	Single arm: National Cancer Institute (NCI)-4650, a messenger ribonucleic acid (mRNA)-based, personalized cancer vaccine	All participants were evaluated as progressive disease (PD) before the trial was terminated
NCT04072900	Melanoma (skin)	Recruiting	Phase 1	30	Single arm: personalized NeoAntigen cancer vaccine-Neo-Vac-Mn	
NCT03532217	Metastatic hormone-sensitive prostate cancer	Active, not recruiting	Phase 1	19	Single arm: PROSTVAC/ ipilimumab/nivolumab/neoantigen DNA vaccine	
NCT03361852	Follicular lymphoma	Recruiting	Phase 1	20	Group A: NeoVax; Group B: NeoVax and pembrolizumab	
NCT03122106	Pancreatic cancer	Active, not recruiting	Phase 1	15	Single arm: personalized neoantigen DNA vaccine	
NCT03956056	Pancreatic cancer	Recruiting	Phase 1	15	Single arm: neoantigen peptide vaccine	
NCT02287428	Glioblastoma	Recruiting	Phase 1	56	Group A: standard RT followed by NeoVax; Group B: pembrolizumab with RT followed by NeoVax + pembro.	
NCT03199040	Triple negative breast cancer	Active, not recruiting	Phase 1	18	Group A: neoantigen DNA vaccine + durvalumab; Group B: neoantigen DNA vaccine	
NCT03422094	Glioblastoma	Terminated	Phase 1	3	Single arm: NeoVax + Nivolumab	
NCT03219450	Lymphocytic leukemia	Recruiting	Phase 1	15	Group A: NeoVax; Group B: Neovax + low-dose cyclophosphamide + pembrolizumab	
NCT04864379	Advanced malignant solid tumor	Recruiting	Phase 1	30	Group A: RFA + PD-1 + iNeo-Vac-P01; Group B: RFA + iNeo-Vac-P01 + PD-1	

Table 1 (continued).

NCT Number	Patients	Status	Phases	Number of enrolled patients	Arms and interventions	Results
NCT04105582	Breast cancer; triple negative breast cancer	Active, not recruiting	Phase 1	5	Single arm: neo-antigen pulsed dendritic cell	
NCT04015700	Glioblastoma	Recruiting	Phase 1	12	Single arm: vaccine (GNOS-PV01 + INO-9012)	
NCT04161755	Pancreatic cancer	Active, not recruiting	Phase 1	29	Single arm: atezolizumab, RO7198457, mFOLFIRINOX	

AJCC v8: American Joint Committee on Cancer version 8; DNAJB1: DnaJ homolog subfamily B member 1; PRKACA: Protein kinase cyclic adenosine monophosphate-activated catalytic subunit alpha; EGFR-TKI: Epidermal growth factor receptor-tyrosine kinase inhibitor; 6MHP: 6 Melanoma helper peptide vaccine; mFOLFIRINOX: Leucovorin+5-fluorouracil+irinotecan+oxaliplatin; PD-1: Programmed death-1; PD-L1: Programmed death ligand 1; RFA: Radiofrequency ablation; RT: Radiotherapy.

methods to identify neoantigen candidates, which are then pulsed into the engineered B cells for antigen presentation. The B cells are cultured with tumor cells and TILs, which are isolated for identifying the neoantigens and TCRs.[36] DLpTCR, a multimodal ensemble deep learning framework, is able to predict the likelihood of interaction between single/paired chains of TCR and peptide presented by MHC.[37] DeepImmuno is a deep learning based method able to predict immunogenic peptides for T-cell immunity.[38] However, no consensus approaches have been established by mathematical and statistical models, and there are great variations among neoantigen identification methods. The general workflows are quite similar and mainly include pre-processing of raw data, read alignment, somatic mutation calling, human leukocyte antigen (HLA) allele typing, peptide inference, peptide-MHC binding prediction, TCR-peptide-MHC complexes (pMHC) interaction estimation, and *in vitro* immune screening. The general approaches for neoantigen identification and applications have been extensively reviewed elsewhere.[39-41] In this review, we summarize the state-of-the-art computational tools for neoantigen analysis and provide an extensive discussion of critical concepts and practical guidance for each analysis step [Figure 2].

Somatic variant calling

The pre-requisite for inferring neoantigens is the selection of appropriate tools for the identification of somatic variants. A large variety of mutation types can produce neoantigens in cancer, including single nucleotide variants (SNVs), short insertions and deletions (INDELs), gene fusions, exon-exon junctions, intron retentions, and alternative splicing events. Numerous somatic variant callers are applied to DNA sequencing data (whole genome sequencing [WGS], whole exome sequencing [WES], or targeted amplicon data) of tumor and matched non-tumor samples to identify somatic mutations including SNVs and INDELs. However, the sensitivity and false positive rates of these methods are highly variable, and this leads to substantial differences among called variants.[42] Popular SNV calling methods include SAMtools, VarScan2, MuTect, VarDict, SomaticSniper, and Strelka.[43] MuTect2, Strelka, GATK, SAMtools, VarScan, and VarDict are also able to call INDELs while Pindel is specifically designed for large INDEL calling.[44] For high-allelic-fraction somatic SNV calling, the impact of caller selection is generally weak. MuTect and Strelka have high sensitivity for detecting low-allelic-fraction SNVs. VarScan2 and SomaticSniper provide less sensitivity for calling somatic SNVs of the low-allelic-fraction.[45] VarScan2 sensitivity can be improved by tuning the minimum allele fraction threshold from the default value of 0.2 to a lower value but at the cost of significantly compromised specificity.[45] SAMtools with default settings reports any possible changes in nucleotides with low specificity and with a high false-positive rate. VarDict calls a few more sites than expected in the benchmark data, which suggests its low sensitivity in SNV calling. Multiple callers and repeats significantly decreased false-positive calls for SNVs and INDELs. A manual review of somatic mutations from callers in the Integrative Genomics Viewer

can further reduce false positives. Gene fusions can be detected from RNA sequencing (RNA-seq) data using various tools including FusionHunter, FusionMap, MapSplice, TopHat-Fusion, BreakFusion, SOAPfuse, EricScript, and FusionCatcher.[46] The performance of gene fusion detection tools largely depends on the quality, read length, and number of reads of RNA-seq data.[47] Overall, neoantigen identification requires a sensitive, accurate, and comprehensive somatic variant calling pipeline that can robustly detect all variant classes that are relevant to a tumor type.

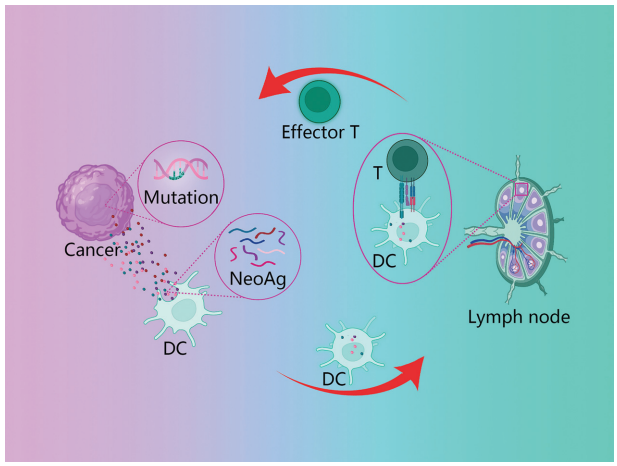

Figure 1: T cell response induced by neoantigens. DC: Dendritic cell; NeoAg: Neoantigen; T: T cell.

HLA allele typing

T cells recognize neoantigens presented by APCs on MHC I or II molecules, which are encoded by the HLA gene complex located in highly polymorphic regions of chromosome 6p21.3 and have over 12,000 alleles.[48] As HLA genes are unique to each individual, it is essential to have accurate HLA haplotyping for neoantigen prediction. Sequence-specific polymerase chain reaction (PCR) amplification, which is laborious and expensive, is the gold standard for clinical HLA typing. Computational HLA typing is becoming a popular approach and uses patients' WGS, WES, or RNA-seq data from a peripheral blood or skin sample. Multiple tools including seq2HLA, PHLAT, HLAMatchmaker, HLAreporter, HLAforest, HLAminer, and xHLA were developed to precisely type class I HLA.[41]

However, class II HLA typing is still challenging and unreliable, with a few benchmarking studies reporting that PHLAT, HLA-VBSeq, seq2HLA, xHLA, and HLA-HD show comparable accuracies with WES and RNA-seq data.[49] Due to the varying capturing efficiency of DNA from HLA genes, it is critical to carefully examine the read coverage from WES/RNA-seq for HLA genes or to construct ensemble methods to produce optimal prediction.[50,51]

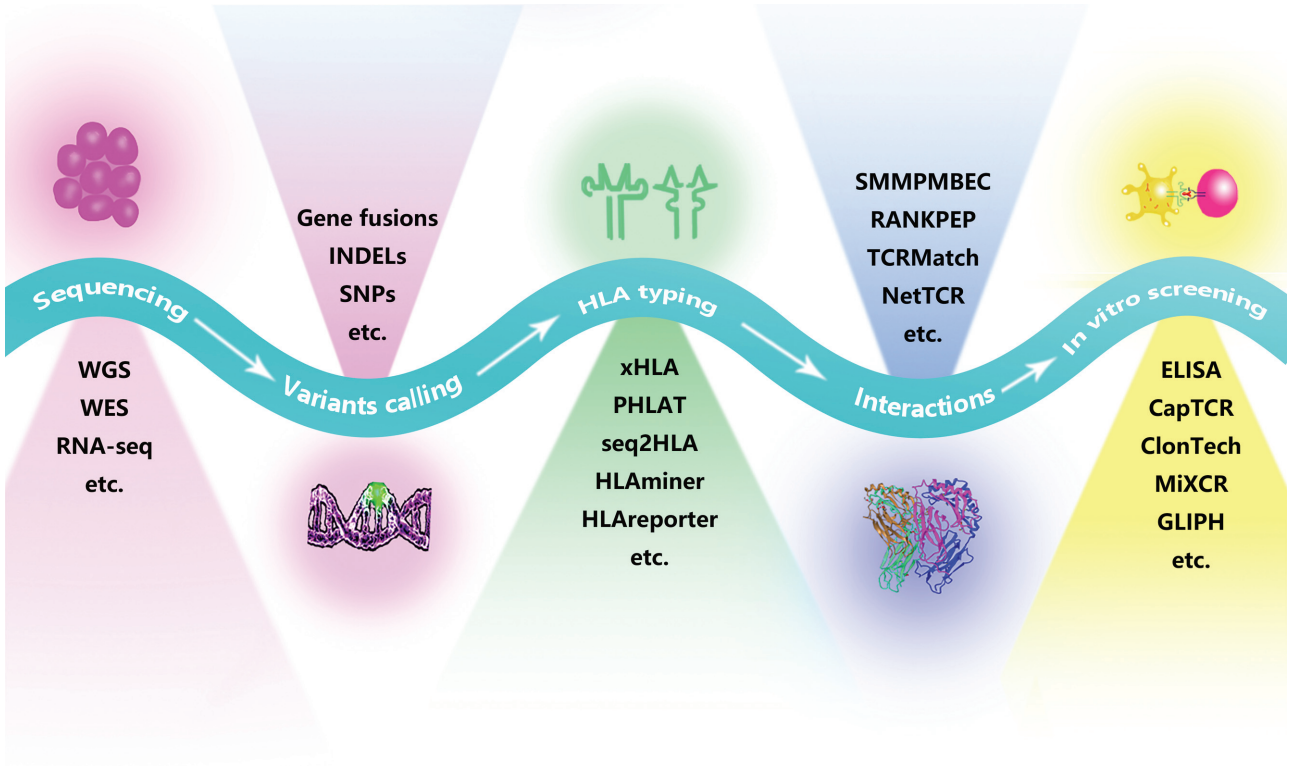

Figure 2: The workflow of neoantigen identification. The whole workflow can be divided into five steps. The first step is sequencing of the cancer cells by WGS, WES or RNA-seq, *etc.* The following steps are variants calling, HLA typing and interactions prediction (interactions among HLA-neoantigen-TCR) based on the sequencing data. The last step is screening the interactions *in vitro*. ELISA: Enzyme-linked immunosorbent assay; GLIPH: Grouping of lymphocyte interactions by paratope hotspots; HLA: Human leukocyte antigen; INDELs: Short insertions and deletions; RNA-seq: RNA sequencing; SNPs: Single nucleotide polymorphisms; TCR: T cell receptor; WES: Whole exome sequencing; WGS: Whole genome sequencing.

Neoantigen HLA-class I allele interactions

About 8–11 N- and C-terminal peptide residues are involved in binding with MHC class I molecules, which then present the peptides to cytotoxic $CD8^+$ T lymphocytes to elicit T cell immunity.[52] It is necessary to predict the binding affinity of mutant peptides to HLA-class I alleles to prioritize mutant peptides. A few computational methods have been developed to evaluate the binding affinity between HLA-class I alleles and mutant peptides and include scoring function-based methods, machine learning-based tools, and consensus methods. The performances of HLA-class I-peptide binding prediction methods have reached a high level (area under the receiver operating characteristic curves [auROC] > 0.95). Scoring function-based methods prioritize mutant peptides by calculating sequence features including sequence similarity, amino acid frequencies, position-specific scoring matrices (PSSMs), and BLOSUM matrices. SYFPEITHI, RANKPEP, PickPocket1.1, SMMPMBEC, PSSMHCpan 1.0, and MixMHCpred 2.0.1 are scoring function-based approaches.[53] Machine learning tools such as NetMHC 4.0,[54] NetMHCstabpan 1.0,[55] NetMHCpan 4.0,[56] ConvMHC,[57] HLA-CNN[58] and MHCflurry 1.2.0[59] assign a mutant peptide as a binder or non-binder by constructing a training model using extracted representative features. Consensus-based tools such as NetMHCcons 1.1[60] integrate several peptide-MHC binding prediction methods in a weighted manner. The most widely used is NetMHC-pan 4.0, which uses artificial neural networks trained on a combination of more than 85,0000 quantitative binding affinities and mass-spectrometry-eluted ligand peptides.

Neoantigen HLA-class II allele interactions

Neoantigens are presented by MHC class II molecules to $CD4^+$ T cells or "helper T cells", which may then stimulate humoral or cell-mediated immune response pathways.[61] Precise prediction of peptide-MHC interactions is important for identifying neoantigens. Unlike MHC class I molecules, class II molecules, which include an α-chain and a β-chain, have higher variability with an open binding pocket on both ends that allows a larger range of peptides to bind. The peptides that bind to MHC class II molecules have a length range of 13 to 25 amino acids. Computational models for MHC class II peptide binding prediction are usually built on matrix-based approaches and ensembles of artificial networks. Popular methods include ARB, MHCPRED, PROPRED, RANKPEP, SMM-align, SVMHC, SVRMHC, SYFPEITHI, MultiRTA, and NetMHCIIpan.[51] A very recent transformer neural network model, BERTMHC, was reported to outperform other models[62,63] Consensus methods that combine multiple models may help improve performance, but rigorous ongoing efforts are needed to further improve the effectiveness of existing models.

TCR-pMHC interaction

Not all peptides presented by MHC molecules are immunogenic. TCRs must contact both the peptide and MHC molecules to elicit an immune response. There are remarkable similarities in the topology of TCR binding to pMHC irrespective of MHC class I or class II restriction. TCR-pMHC interactions can help further narrow down true neoantigens out of MHC-presented peptides. Very recently, a number of machine learning computational methods constructed based on complementarity determining region 3 (CDR3) sequences or additional cellular information such as SETE,[64] ERGO,[65] NetTCR-2.0,[66] and TCRMatch[67] have been reported to predict TCR-pMHC interactions. However, the prediction models of TCR-pMHC interactions have poor performance owing to the limited availability of training data. There is an urgent need for the development of cost-effective and accurate computational methods to assess TCR-pMHC binding.

In vitro immune screening

In vitro screening of T cell responses triggered by candidate neoantigens may provide direct evidence that a given neoantigen is immunogenic. T cell responses elicited by neoantigens may cause clonal expansion of T cells, upregulation of activation markers on the cell surface, effector cell differentiation, cytotoxicity induction, or cytokine secretion. A popular approach is to assess the activation of T cells in peripheral blood mononuclear cells by neoantigens *in vitro* (generally 2–4 days of incubation). An alternative method for *in vitro* T cell stimulation is to use *Escherichia coli* that expresses predicted neoantigens in autologous APCs, which are then incubated with T cells derived from the patient's PBMCs. T cells are then harvested and processed for various assays. Production of cytokines such as tumor necrosis factor α and interferon γ can be measured through enzyme-linked immunospot assay[68] or intra-cellular cytokine staining. Additionally, the clonality and diversity of the TCR repertoire suggest whether neoantigen-triggered T cell responses have occurred,[69] and TCR clonotyping (identification and characterization of CDR3 sequences of T cell clones) has been used to identify clonal T cell responses to neoantigens. CDR3 sequences can be identified using output data from focused assays such as Adaptive, ClonTech, or CapTCR bulk tissue RNA-seq[70] and single-cell RNA-seq.[71] It is essential to characterize the diversity of the repertoire, determine the pairing of TCR α (TRA) and TCR β (TRB) clonotypes, and pair T cell clones with their target neoantigens. MiXCR (http://mixcr.milaboratory.com/https://github.com/milaboratory/mixcr/) was developed to extract TCR sequences from raw data of both bulk and single-cell sequencing and then group them into identical clonotypes.[72] MIGEC[73] was designed for methods using unique molecular identifiers, and TraCeR,[71] which is specific for single-cell methods, is able to identify paired α–β sequences derived from the same clonally expanded cells. Oligoclonal T cell populations with consistent CDR3 motif sequences are reported to be able to recognize the same neoantigen,[74] therefore, it is possible that there is one-to-one mapping between T cell clones and neoantigens. Grouping of lymphocyte interactions by paratope hotspots can identify CDR3 motifs across T cells in bulk TCR sequencing.[75] Collectively, *in vitro* immune screening can further filter out immunogenic neoantigens, which have

the potential to improve the survival of patients with cancer.

Clinical Applications of Neoantigens

Biomarkers for immunotherapy

To optimize the efficiency of immunotherapy, researchers have investigated numerous prognostic biomarkers such as tumor mutation burden, microsatellite instability, mismatch repair deficiency, TILs, and programmed death ligand 1 (PD-L1) expression. Recently, more attention has been paid to a new biomarker, the neoantigen. Lauss *et al*[76] showed that a higher neoantigen load is associated with better progression-free survival and overall survival (OS) of patients with melanoma treated with adoptive T-cell therapy. Another study revealed that specific neoantigens contribute to the long-term survival of patients with pancreatic cancer.[33] Furthermore, the investigators observed the selective loss of neoantigens with metastatic progression, which implied that neoantigens could be used as biomarkers for disease progression and patient survival. Similarly, Ren *et al*[77] reported that neoantigens can be used to predict OS independent of TILs and other biomarkers in breast cancer. They showed that higher HLA-I- or HLA-II-restricted neoantigen load correlated with better OS. For patients with adenoid cystic carcinoma (ACC), researchers found that a low neoantigen load was correlated with a poor response to anti-PD1 therapy and a high cancer recurrence rate. The investigators suggested that successful immunotherapy for ACC requires a "hot" immune microenvironment, namely, more TILs and neoantigens. Therefore, neoantigens can be utilized as positive prognostic biomarkers similar to TILs. However, Quintana *et al*[78] showed that tumors with more TILs have lower neoantigen loads. Contrary to previous studies, their results hint at a negative correlation between TILs and neoantigens. Their hypothesis is that failure of lymphocytes to migrate into the tumor leads to the survival of tumor cells and neoantigens. Thus, the negative correlation between TILs and neoantigens suggests that neoantigens can be used as negative prognostic biomarkers, contrary to TILs. The reason why they reach a conclusion different from others may be that their data are not comprehensive because they exclude patients with TILs percentage between 5% and 50%, who may be the major group who need to be considered. Furthermore, Balachandran *et al*[33] revealed that it is the quality rather than the quantity of neoantigens that correlate significantly with prognosis. Similarly, Rosenthal *et al*[79] proved that high clonal neoantigen burden was associated with better prognosis while the sub-clonal or total neoantigen burdens were not. Therefore, we can evaluate the prognosis before and during immunotherapy by tracking high-quality neoantigens. Our previous work showed that high-quality neoantigens, tracked by circulating tumor DNA (ctDNA) sequencing, can be used to predict the prognosis of patients with non-small cell lung cancer (NSCLC).[80] Specifically speaking, because of the high heterogeneity of somatic mutations among patients, it is infeasible to track neoantigens by ctDNA sequencing using common gene panels, which would be far too complicated and costly. Thus, we proposed the method of using the individually customized panels to track neoantigen evolution during ICB treatment [Figure 3]. Our method is proved to be more sensitive, economical and feasible in clinical practice, making sequencing-based personalized management in immunotherapy possible.

Therapies based on neoantigens

Therapies based on neoantigens can be divided into two types: neoantigen vaccines and neoantigen-specific T-cells. Neoantigen vaccines include four main categories: peptide vaccines, mRNA vaccines, DNA vaccines, and DC vaccines. Each category has its own technical route and clinical characteristics [Figure 4].

Peptide vaccines based on neoantigens are widely used in clinical practice. Ott *et al*[9] synthesized 20 peptides that target personal tumor neoantigens. Vaccinated with these peptides, 80% (4/6) of melanoma patients survived without recurrence for 25 months. The other 2 patients who experienced recurrence achieved complete recession after subsequent anti-PD-1 therapy. The investigators discovered that peptide vaccines can activate pre-existing neoantigen-specific T cells and induce a wide range of new specific T cells. In a glioblastoma trial, researchers have shown that neoantigens can ignite specific circulating $CD4^+$ and $CD8^+$ T cell responses and increase TILs. This trial also proved that circulating neoantigen-specific T-cells can be recruited to intra-cranial tumors.[30] Mueller *et al*[81] revealed that a H3.3K27M-specific vaccine could induce neoantigen-specific T cell responses in 39% (7/29) of participants. Patients with a positive response had a significantly longer OS than the others. Recently, another study showed that it is feasible and safe to combine peptide vaccines with anti-PD-1 therapy in melanoma, NSCLC, and bladder cancer. The 1-year OS rates were 96%, 83%, and 67% for patients with melanoma, NSCLC, and bladder cancer, respectively, which were better than the historical data from anti-PD-1 monotherapy studies.[82]

Kreiter *et al*[83] showed that neoantigen mRNA vaccines can induce cytotoxic T lymphocyte responses and reshape the tumor microenvironment associated with tumor control in mice. Furthermore, Cafri *et al*[84] designed an mRNA vaccine encoding up to 20 neoantigens for patients with gastrointestinal cancer. The mRNA vaccine has been shown to safely elicit neoantigen-specific T cell responses. Unfortunately, the investigators observed no objective responses among the four patients in the trial. As mentioned by other researchers, instability may be a critical obstacle for mRNA vaccines.

DNA has also been used to produce neoantigen vaccines. Researchers have shown that synthetic DNA vaccines – recombinant plasmids that can produce neoantigens – can generate robust T cell responses in mice.[85-87] Another study indicated that combining DNA vaccination with anti-PD-1 therapy can achieve a synergistic effect in controlling tumor growth.[88] Their data suggest that a combination strategy of immunotherapies may bring about unexpected benefits. In a pre-clinical model, Li *et al*[89] revealed that

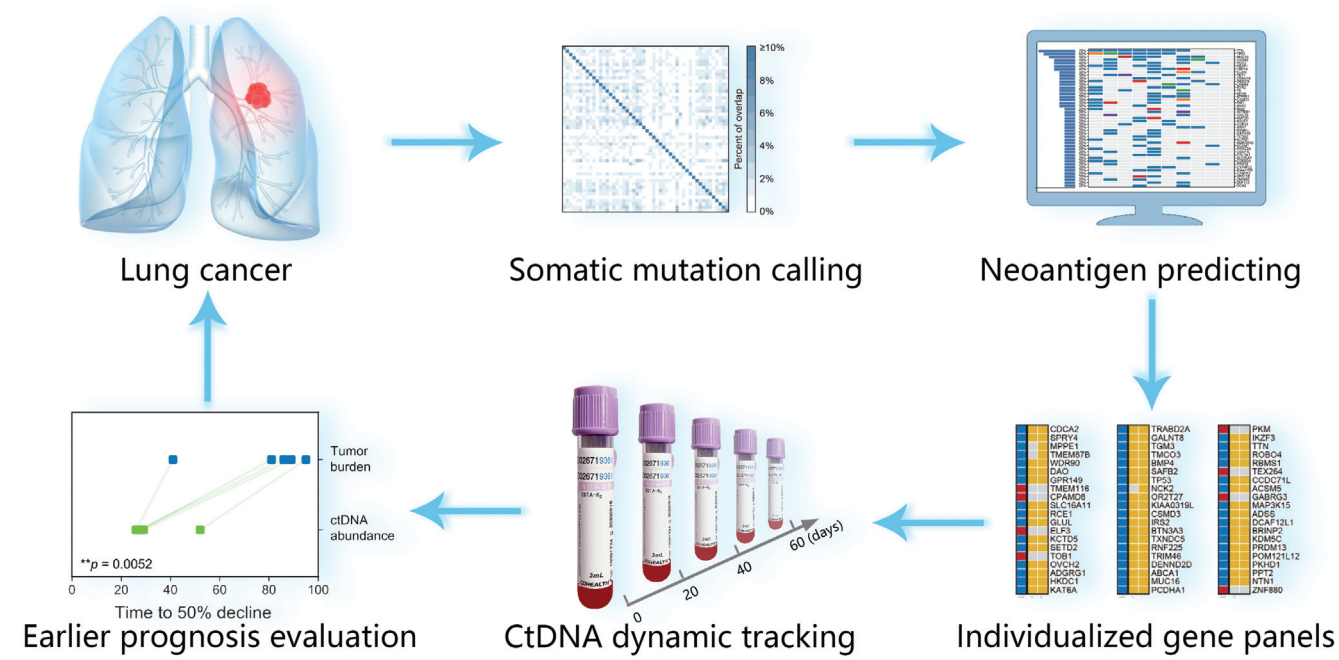

Figure 3: Dynamic neoantigen tracking is used to evaluate the prognosis of cancer patients. This evaluation strategy can be more sensitive and reliable than imaging examination. Individualized gene panels are the key point to make this strategy sensitive and economical. ctDNA: Circulating tumor DNA.

optimized neoantigen DNA vaccines are capable of inducing specific T cell responses in patients with pancreatic cancer. More clinical trials are needed to verify the effectiveness and safety of neoantigen DNA vaccines.

Neoantigen-pulsed DCs have been shown to promote the presentation of HLA class I-restricted neoantigens and amplify neoantigen-specific T-cells.[69] Furthermore, Dillman *et al*[90] utilized DCs that were pulsed by autologous tumor antigens to vaccinate 54 patients with metastatic melanoma. These patients achieved a longer median survival and lower risk of death. The investigators chose to use whole tumor antigens instead of well-selected neoantigens to pulse the DCs but still obtained satisfying results. However, as is widely accepted, most tumor antigens are not immunogenic. Therefore, we suppose that the real effective antigens mixed in the whole tumor antigens may be neoantigens. In a phase Ib study, researchers

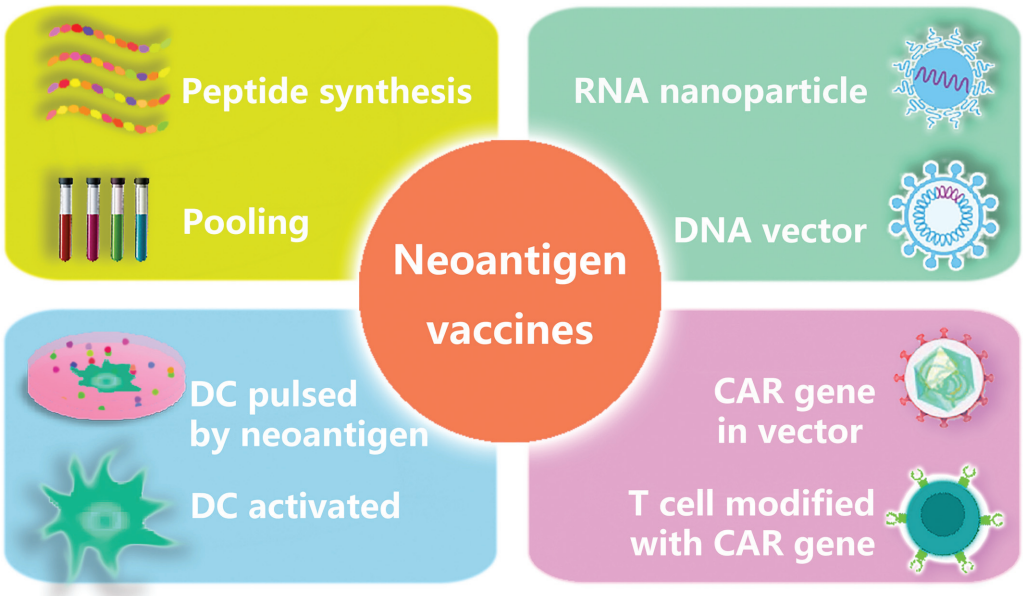

Figure 4: Therapies based on neoantigens. CAR: Chimeric antigen receptor; DC: Dendritic cell.

produced a DC vaccine pulsed with a personalized peptide. This neoantigen-pulsed DC vaccine has been shown to amplify the clinical effect of ICB therapy in patients with pancreatic adenocarcinoma, especially in those lacking TILs.[91] Neoantigen-pulsed DC vaccines can directly present neoantigens to T cells. However, their manufacture is time- and resource-consuming.

Adoptive cell therapy (ACT) is also a mainstream immunotherapy that shows surprising therapeutic effects for B cell malignancies. Moreover, it is a promising strategy to integrate ACT with neoantigens. To date, ACT can be roughly divided into three approaches: TILs, chimeric antigen receptor T cells (CAR-T), and TCR engineered T cells. TILs are isolated from the patient's tumor and expanded *ex vivo*. By selecting neoantigen-specific TILs to expand, large amounts of T cells with high competence can be harvested, and this contributes to better anti-tumor immunity.[92] CAR-T therapy is famous for its successful application in acute lymphoblastic leukemia and diffuse large B-cell lymphoma. Bajgain *et al*[93] showed that CAR-T cells targeting a mucin 1 neoantigen can expand selectively at the tumor site and achieve durable anti-tumor immunity. Neoantigen-specific TCR gene-engineered T-cells have been shown to be effective in multiple pre-clinical studies.[94-96] Many clinical trials have also shown that these TCR gene-engineered T cells mediate clinically meaningful anti-tumor immunity.[97,98]

Clinical trials of various vaccines targeting at cancer neoantigens are emerging [Table 1]. Though the outcomes are far from satisfying at present, such attemps are highly encouraging. Breakthrough in this aspect will certainly benefit a large number of patients with cancer.

Role in drug resistance

Drug resistance is an inevitable conundrum in anti-tumor therapy. One of the main causes is the emergence of resistant mutations in tumor cells. Such resistant mutations may reactivate targeted pathways, activate collateral pathways, activate drug efflux pumps, or inhibit the apoptosis of cancer cells.[99] However, every coin has two sides. These mutations are perfect candidates for neoantigen screening. By investigating drug-resistant tumors, neoantigens can be discovered more efficiently and economically.[100] Cancer cells expressing such neoantigens are susceptible to the immunotherapies mentioned previously. Therefore, immunotherapies targeting neoantigens can be used to overcome drug resistance. Drug-resistant cancer cells continue to evolve during the course of treatment, similar to neoantigens. Thus, by depicting the evolving neoantigen landscape, we were able to predict the acquired resistance of cancer cells.

Conclusions and Perspectives

Significant progress has been made by researchers worldwide in the past few decades. Our understanding of the immune response induced by neoantigens is becoming deeper and deeper. Workflows for identifying neoantigens are also emerging and rapidly improving. Therapies targeting neoantigens are widely practiced in clinical trials and show satisfactory safety, specificity, and tolerability. However, there is still a long way to go before neoantigen therapies become the first-line choice for doctors. Several critical deficiencies hamper its extensive clinical application. Firstly, workflows of neoantigen identification are often complicated. High throughput DNA sequencing and mass spectrometry are usually needed. In-silico algorithm is also indispensible. *In vitro* and *in vivo* procedures are complex as well. Secondly, the course of neoantigen-based therapy is time-consuming. Most patients do not have that much time waiting for its preparation. Thirdly, the expenditure of this therapy is strickingly high. Except for participating in clinical trials, few patients can afford it.

Admittedly, workflows of neoantigen identification and designs of neoantigen-based therapies are far from satisfying. However, finished clinical trials brought us the signs of the dawn. By simplifying these processes, reducing the cost and elevating accuracy and efficacy, more patients will benefit from precision therapies based on neoantigens in the near future. In addition, combining neoantigen-based precision therapies with other immunotherapies may be an effective strategy to gain unexpected benefit. Therapies activating the antigen presenting cells such as DCs and macrophages are worth trying. Besides, keeping the immune response induced by neoantigen-based therapy lasting longer is also calling for our efforts.

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Conflicts of interest

None.

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