Strategies for neoantigen screening and immunogenicity validation in cancer immunotherapy (Review)

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Abstract. Cancer immunotherapy stimulates and enhances antitumor immune responses to eliminate cancer cells. Neoantigens, which originate from specific mutations within tumor cells, are key targets in cancer immunotherapy. Neoantigens manifest as abnormal peptide fragments or protein segments that are uniquely expressed in tumor cells, making them highly immunogenic. As a result, they activate the immune system, particularly T cell-mediated immune responses, effectively identifying and eliminating tumor cells. Certain tumor-associated antigens that are abnormally expressed in normal host proteins in cancer cells are promising targets for immunotherapy. Neoantigens derived from mutated proteins in cancer cells offer true cancer specificity and are often highly immunogenic. Furthermore, most neoantigens are unique to each patient, highlighting the need for personalized treatment strategies. The precise identification and screening of neoantigens are key for improving treatment efficacy and developing individualized therapeutic plans. The neoantigen prediction process involves somatic mutation identification, human leukocyte antigen (HLA) typing, peptide processing and peptide-HLA binding prediction. The present review summarizes the major current methods used for neoantigen screening, available computational tools and the advantages and limitations of various techniques. Additionally, the present review aimed to summarize experimental strategies for validating the immunogenicity of the predicted neoantigens, which will determine whether these neoantigens can effectively trigger immune responses, as well as challenges encountered during neoantigen screening, providing relevant recommendations for the optimization of neoantigen-based immunotherapy.

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Contents

1. Introduction

As the global population ages and lifestyles change, the burden of cancer continues to increase. According to global cancer statistics, there were nearly 20 million new cancer cases and ~9.7 million cancer-related deaths worldwide in 2022 (1). It is expected that by 2050, the number of new cancer cases will exceed 35 million (2), and cancer will become a notable challenge to global public health. Due to innovations in cancer treatment methods and drugs, the overall mortality rate has decreased. In the United States, recent statistics indicate that approximately 4.5 million cancer-related deaths were averted between 1991 and 2022 (3). However, owing to the involvement of complex factors in tumor development, effective curative methods are still lacking (4). Historically, treatment methods for tumors have primarily included surgery and radiation and drug therapy (Fig. 1). Although surgical treatment can directly remove tumors, it often causes notable trauma and bleeding, and postoperative immunity may decline (5). Additionally, the healing response of wounds can potentially lead to the growth of metastatic tumors (6), resulting in decreased safety. Radiation therapy uses high-energy rays or particles to kill cancer cells with a short treatment duration and generally localized reactions. Drug therapy relies mainly on chemotherapeutic drugs to inhibit or kill cancer cells. Chemotherapy is often used in combination with surgery or radiotherapy to control

tumor growth. However, both radiation and chemotherapy can damage normal cells while killing tumor cells (7) and may also lead to complications (8). For example, both treatment modalities induce mucositis (9) and bone marrow suppression (10,11). Radiotherapy can also cause skin disorders (12) and carries the risk of inducing secondary malignancies (13). Chemotherapy may result in gastrointestinal complications (14), premature ovarian failure and infertility (15). In addition to chemotherapeutic drugs, targeted therapeutic and immunotherapeutic drugs have become an important means of tumor treatment (16,17). Targeted therapy prevents the proliferation of cancer cells by interfering with specific molecules required for carcinogenesis or tumor proliferation (18), whereas immunotherapeutic drugs are safer because they kill tumor cells by inducing or enhancing immune responses (19). Hybrid cell vaccine immunization (20), heat shock proteins (HSP) (21), bacterial extracts (22) and T body therapies (23) are commonly used. Mycomedicine (24) is an immunotherapeutic strategy and their metabolites are regarded as key oncological therapeutic drugs because they enhance immune responses by inducing apoptosis or autophagy and decreasing tumor metastasis. In recent years, with the rapid development of sequencing technology and immunology, tumor immunotherapy has gradually become an emerging tumor therapeutic approach (25,26).

Cancer originates from the abnormal proliferation of normal cells. Cancer cells are similar to normal cells in terms of morphology and biological characteristics, making it difficult for the immune system to recognize and attack them (27,28). When tumor cells are mistakenly identified by the immune system as self-components, they evade immune surveillance, allowing them to grow and spread. To address, researchers have explored various methods aimed at enhancing the immune system antitumor response and improving its ability to suppress tumors (29-31). Tumor immunotherapy actively or passively activates the immune system to generate tumor-specific immune responses, thereby inhibiting or killing tumor cells. Unlike traditional treatment methods, immunotherapy does not directly kill cancer cells; instead, it enhances the immune system and mobilizes immune cells to recognize and kill tumor cells. This results in higher specificity and efficiency and fewer side effects (32). Numerous types of tumor immunotherapy have been developed, including monoclonal antibody therapy (33), immune checkpoint inhibitors (ICIs) (34), tumor vaccines (such as Provenge and Cimavax) (35,36), adoptive cell therapy (37) [such as T cell receptor (TCR) therapy (38,39), chimeric antigen receptor (CAR) T cell therapy (CAR-T) (40), and chimeric antigen receptor natural killer (CAR-NK) cell therapy (41)] and non-specific immune modulators. Among these, peptide-based neoantigen vaccines are gradually becoming popular in the field of tumor immunotherapy owing to their unique advantages (42,43): Neoantigen vaccines can accurately identify tumor cells and enhance tumor-specific immune responses, thereby achieving the targeted destruction of tumors (44). In addition, neoantigen vaccines stimulate long-term immune memory, decreasing the risk of tumor recurrence and metastasis (45), making them a highly promising option for personalized treatment.

2. Tumor neoantigen vaccines

Research on tumor immunology can be traced back to the early 20th century. Paul Ehrlich proposed the concept of immune surveillance', suggesting that the immune system is capable of recognizing and suppressing spontaneously arising tumor cells, thereby preventing their progression into detectable malignancies (46). This hypothesis laid the groundwork for future research in tumor immunology. In 1943, Gross was the first to demonstrate that methylcholanthrene-induced sarcomas could elicit an immune response in mice (47). In 1966, Baldwin (48) confirmed the recognition and rejection response of the immune system to spontaneous tumors, providing a theoretical foundation for the study of tumor antigens.

The unique advantage of tumor immunotherapy stems from its ability to fully utilize the selective recognition and attack mechanisms of the human immune system, thereby specifically killing tumor cells without damaging healthy cells (49). This mechanism relies on the inherent antitumor characteristics of the human adaptive immune system in which $CD8^+$ and $CD4^+$ T cells serve crucial roles. After antigen recognition, T cells signal tumor cells displaying the antigen to undergo cell cycle arrest and cell death by recognizing peptide fragments on the cell surface via major histocompatibility complex (MHC) class I and II molecules and release paracrine signals to elicit an antitumor response (50). Currently, neoantigen vaccines used in clinical applications primarily include peptide, dendritic cell (DC) and RNA vaccines (51-53), which are directly injected into patients subcutaneously or into the lymph nodes. Peptide vaccines are made directly from neoantigen peptides; after vaccination, the neoantigens can bind to the corresponding human leukocyte antigen (HLA) on DC cells in the body, presenting them to T cells and inducing an immune response (42). DC vaccines use DCs loaded with neoantigen peptides to present neoantigens directly to T cells (52). RNA vaccines involve the injection of RNA fragments that encode neoantigen peptides, which are translated into neoantigen peptides in the body and presented by HLA (54).

Since the beginning of the 21st century, research on tumor antigens has advanced rapidly, with breakthroughs in the immunotherapy of melanoma (55-57). Ott *et al* (58) predicted individual tumor neoantigen vaccines in tests with patients suffering from malignant melanoma and found that these neoantigen vaccines induced responses in 16% of $CD8^+$ T cells and 60% of $CD4^+$ T cells. Among six vaccinated patients, four had no recurrence at 25 months post-vaccination, whereas two patients who experienced disease progression underwent complete tumor regression after receiving anti-PD-1 treatment, and their neoantigen-specific T cell repertoire was expanded. Tumor neoantigen vaccine NeoVax demonstrates long-term efficacy in eight patients with high-risk melanoma (59). Personalized neoantigen vaccination is effective for tumor types beyond melanoma (60). In a previous study, a personalized neoantigen-targeted vaccine was used to immunize patients with newly diagnosed glioblastoma after surgical resection and conventional radiotherapy. Patients who did not receive dexamethasone developed multifunctional neoantigen-specific $CD4^+$ and $CD8^+$ T cell responses that

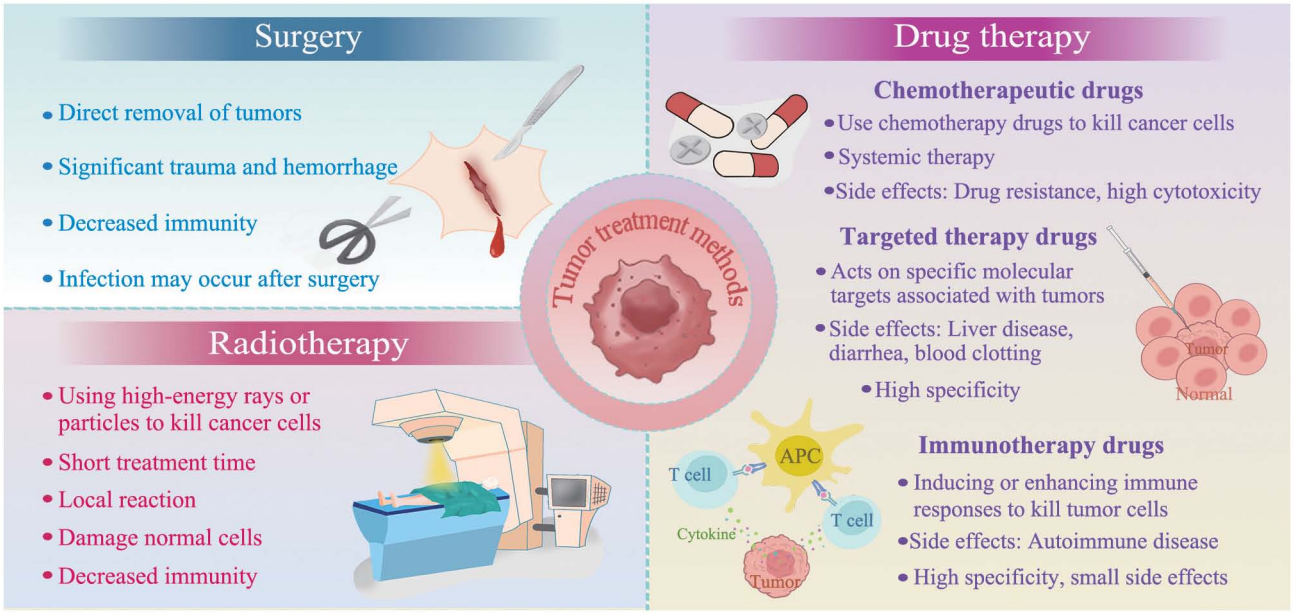

Figure 1. Cancer treatment methods. Cancer treatment primarily includes surgery, radiation therapy, chemotherapy, targeted therapy and immunotherapy. Surgery may directly remove tumors but often involves trauma and bleeding, which can lead to a decline in immune function postoperatively. Improper surgical handling or delayed postoperative treatment lead to tumor recurrence and may even accelerate its metastasis. Radiation therapy uses high-energy radiation to destroy cancer cells, providing localized effects, but may also damage surrounding healthy tissue. Chemotherapy employs cytotoxic drugs to inhibit or eliminate cancer cells and is often used in combination with surgery or radiation therapy, common side effects include drug resistance and high cytotoxicity. Targeted therapy disrupts specific molecular pathways involved in cancer cell proliferation, while immunotherapy enhances the immune response to eliminate tumors, offering a higher level of safety. APC, antigen-presenting cell.

were rich in memory phenotypes and showed an increase in the number of tumor-infiltrating T cells. This demonstrates that neoantigen-targeted vaccines favorably alter the immune environment of glioblastoma. Furthermore, combination therapy with tumor vaccines and immunosuppressive treatment is more effective than single treatments (61,62) and has good prognostic outcomes in the treatment of various types of cancer, including melanoma (63), non-small cell lung cancer (NSCLC) (64) and bladder cancer (65). Notably, Sipuleucel-T (66,67), a therapeutic cancer vaccine, has been approved by the US. Food and Drug Administration for the treatment of asymptomatic or minimally symptomatic metastatic castration-resistant prostate cancer (68). These advancements clearly demonstrate the clinical value and broad potential of cancer vaccines in modern cancer treatment.

3. Neoantigens in tumors

Presentation of neoantigens. In tumor cells, mutated genes are transcribed into mRNA and translated into corresponding mutated proteins. These mutated proteins are degraded by the proteasome into many short peptides (69), which are recognized by antigen-presenting cells (APCs). After entering the endoplasmic reticulum, short peptides are further processed by N-terminal peptidases and bind HLA in tumor cells to form peptide-HLA complexes (pHLA). These pHLA complexes leave the endoplasmic reticulum and are transported to the cell membrane, where they are recognized by TCR and trigger an antitumor immune response (70) (Fig. 2). The mutated short peptides that induce an immune response in tumor cells are referred to as neoantigens.

Sources of neoantigens. The core of cancer immunotherapy is the presence of tumor antigens in tumor cells, which are recognized by the immune system. Based on their specificity, tumor antigens are divided into tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs) (Fig. 3).

Initially, research on tumor vaccines focused on TAAs such as mucin 1 (71,72), human epidermal growth factor receptor 2 (73,74), telomerase reverse transcriptase (75) and tyrosinase (76). However, TAAs are not only selectively expressed or overexpressed in tumor cells but also in some non-tumor cells (77). As the body typically develops immune tolerance to these TAAs, it is difficult to stimulate a strong immune response; thus, immunotherapy targeting TAAs may be ineffective. In addition, targeting TAA may cause the immune system to attack normal tissue, which in turn may trigger autoimmune responses and diseases (78), making TAA not an ideal therapeutic target (79,80).

By contrast, TSA originates from mutations in the cancer genome, is present only in tumor cells and is virtually absent in normal tissues, making it highly tumor-specific. TSA also has greater immunogenicity and better MHC affinity and is not affected by central immune tolerance (81). Because normal tissue cells do not express TSA, immunotherapy targeting TSA does not cause off-target damage to non-tumor tissue and has a better safety profile (82). Therefore, TSA, as a tumor neoantigen, shows potential for clinical applications.

The generation of neoantigens is typically associated with genomic mutation and the number and types of mutation vary according to the cancer type (83,84), including single nucleotide variants (SNVs), insertions/deletions (INDELs) (85) and gene fusion (86,87). Point mutations account for 95% of tumor mutations, whereas INDELs and frameshift mutations

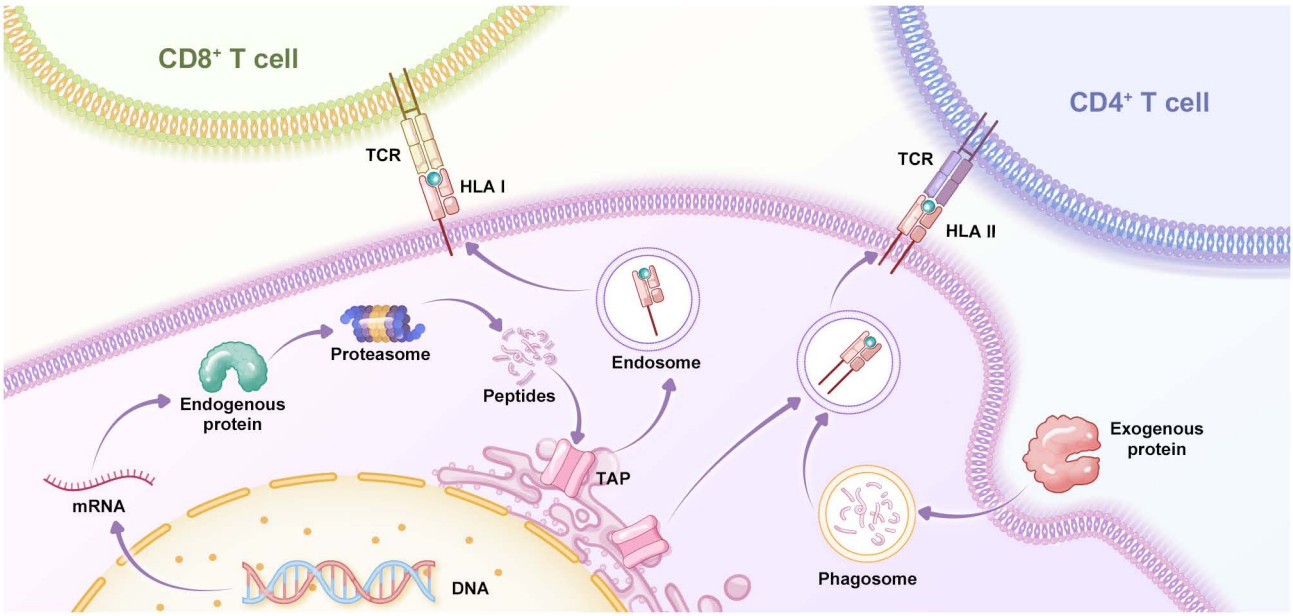

Figure 2. Neoantigen presentation. In tumor cells, mutated genes undergo transcription and translation to produce mutant proteins, which are degraded into short peptides by the proteasome. These peptides are transported into the endoplasmic reticulum, where they undergo processing by N-terminal peptidases and bind HLA molecules to form pHLA. The pHLAs are transported to the cell surface, where a subset can be recognized by TCRs, triggering an anti-tumor immune response. The mutation-derived peptides capable of eliciting immune responses are referred to as neoantigens. HLA, human leukocyte antigen; pHLA, peptide-HLA; TCR, T cell receptor; TAP, transporter associated with antigen processing.

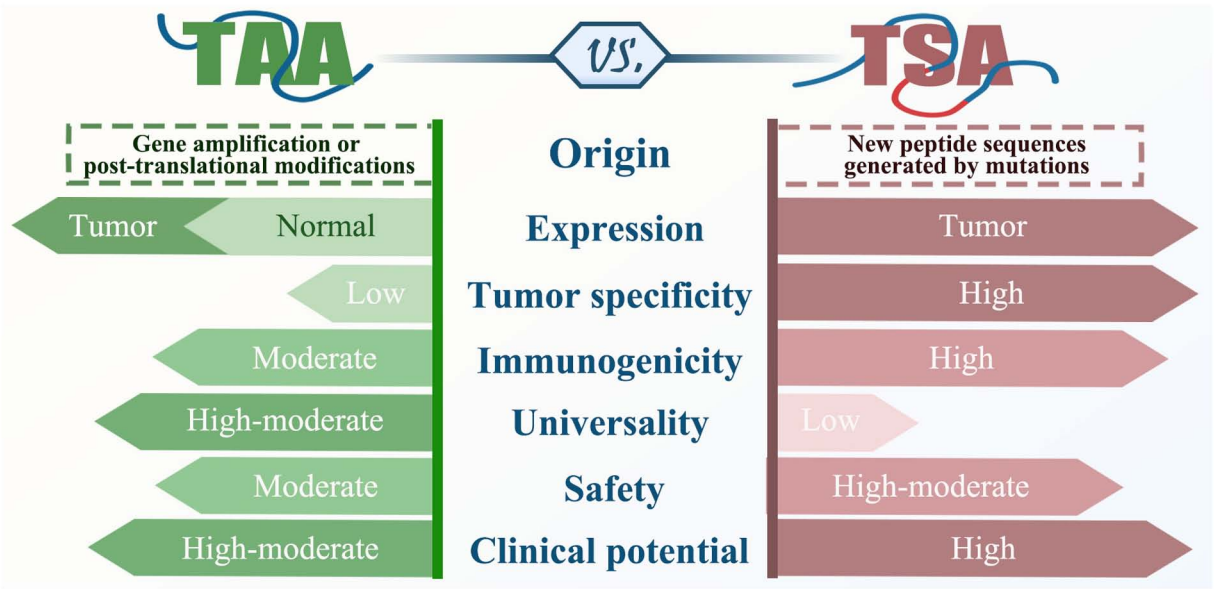

Figure 3. Classification and characteristics of tumor antigens. Tumor antigens are categorized into TAAs and TSAs. TAAs are expressed in both tumor cells and certain normal cells, making them prone to inducing immune tolerance and potentially triggering autoimmune diseases. As a result, they exhibit lower tumor specificity and safety. TSAs are exclusively expressed in tumor cells, demonstrating higher immunogenicity without being affected by immune tolerance. This makes TSAs a safer and more promising target for clinical applications. TAA, tumor-associated antigen; TSA, tumor-specific antigen.

constitute the remaining portion (88). Changes in amino acid sequences and spatial structures caused by INDELs, or frameshift mutations are more pronounced, and mutated peptides have a stronger affinity for MHC, making them more likely to be recognized by T cells as neoantigens (89). Therefore, research has mainly focused on SNVs and INDELs (90), which frequently occur in tumor cells and trigger abnormal protein translation under certain conditions (such as altered RNA splicing (91-93) or imbalances in translational regulation (94)), leading to the formation of neoantigens. Trials of neoantigen vaccines for melanoma indicate that neoantigens derived from SNVs expand T cell populations (95) and induce disease regression (58,96). Although neoantigens generated from SNVs and INDELs have been widely studied (90,97,98), their clinical applications remain limited. These neoantigens often have high patient specificity (99-101), meaning that the mutation may not be the same across patients, resulting in poor universality of neoantigen vaccines and limited

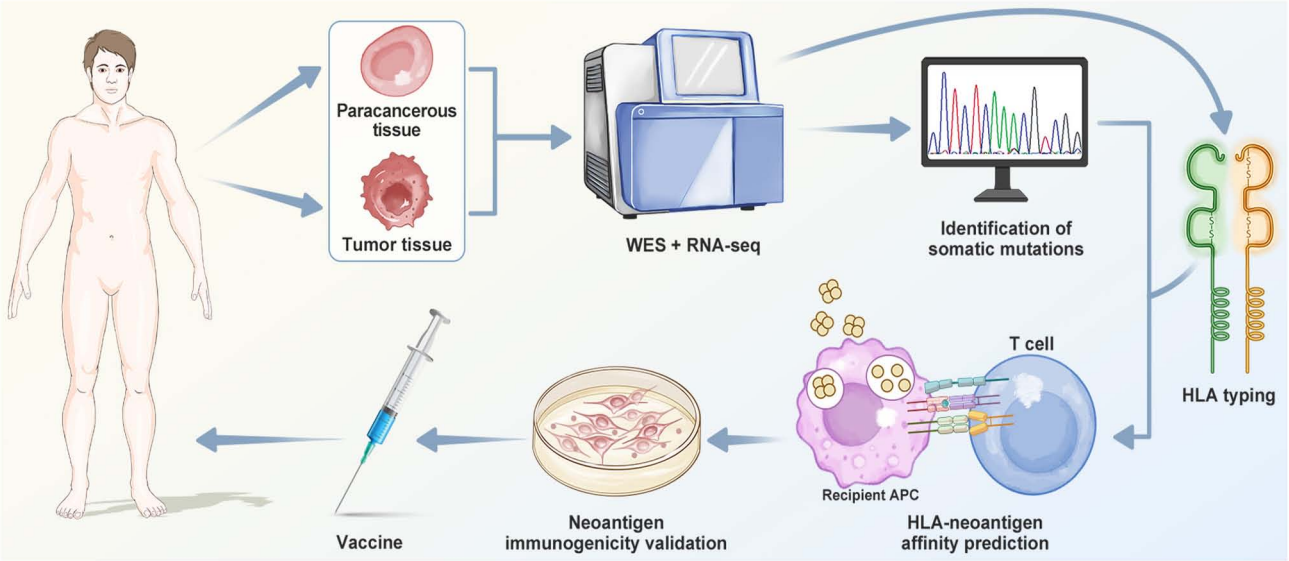

Figure 4. Neoantigen vaccine development. The process of neoantigen vaccine development begins with WES and RNA-seq of both tumor and adjacent normal tissues from the patient to identify somatic mutations. Bioinformatics analyses are conducted to screen for candidate neoantigens, which are refined by predicting their binding affinity to the patient HLA alleles. Immunogenicity of the selected candidate neoantigens is assessed to determine their potential to elicit an effective anti-tumor immune response. Highly immunogenic neoantigens are formulated into a personalized neoantigen vaccine, which is administered to the patient to activate a tumor-specific immune response, thereby exerting anti-tumor effects. WES, whole exome sequencing; seq, sequencing; HLA, human leukocyte antigen; APC, antigen-presenting cell.

clinical efficacy. Fusion gene-derived neoantigens have higher immunogenicity (102). Wei *et al* (103) collected 6,552 tumor samples from The Cancer Genome Atlas (TCGA), including colon adenocarcinoma, stomach adenocarcinoma, uterine corpus endometrial carcinoma, melanoma and predicted a total of 67,502 fusion neoantigens. Compared with SNVs and INDELs, fusion genes can generate up to 6-fold more candidate neoantigens and up to 11-fold more specific candidate neoantigens. Moreover, 5.8% of the fusion neoantigens in TCGA are shared between patients. However, such fusion genes are relatively rare events (104). Splice variants, endogenous retroelements (105) and other tumor-specific processes may also generate neoantigens. In addition to neoantigens derived from mutations in protein-coding genes, peptides produced from non-coding regions have immunogenic potential. Non-coding transcripts can be created from non-coding exons, introns and untranslated regions, as well as non-canonical reading frames within coding regions (106). Laumont *et al* (107) discovered numerous antigens from non-coding regions, including both mutated and unmutated antigens, by studying traditional non-coding sequences in patients with leukemia and lung cancer. Huang *et al* (108) found that peptides encoded by circular RNA (circRNA) not only exhibit immunogenicity but can also effectively induce specific T cell responses, thereby triggering a strong antitumor immune response. These findings provide novel ideas for tumor immunotherapy, suggesting the development of vaccines or immunotherapy strategies using circRNAs and peptides encoded within tumor cells.

4. Neoantigen screening process

Neoantigen screening is a key step in tumor immunotherapy to identify antigens that can effectively activate immune responses and target tumor cell-specific mutations. In 2012, a study of the exome of mouse tumors demonstrated that whole-exome sequencing (WES) could be used to identify neoantigens (36), marking a development in the screening of tumor-specific neoantigens based on genomic analysis. In 2013, Robbins (109) used exome sequencing to identify mutated proteins expressed in patients. The aforementioned study used an MHC molecule-antigen epitope affinity algorithm for simulated predictive assessment and synthesized candidate antigen epitopes to validate the immune response. This method can quickly identify mutated antigens expressed in tumor cells that can be recognized by tumor-infiltrating lymphocytes. In 2017, Sahin *et al* (96) identified individual mutations using exome sequencing technology and designed and synthesized personalized tumor vaccines for patients with melanoma. The results showed that all subjects who received the vaccine developed a strong and efficient specific antitumor T cell response, with two of five patients with metastatic tumors exhibiting objective responses following vaccination. To the best of our knowledge, the aforementioned study was the first time a personalized mutant vaccine has been applied in humans, providing a new path for neoantigen immunotherapy. Exome sequencing technology is widely used in cancer immunology research and clinical practice because of its advantages such as large sequencing data volume, high sequencing depth, mature sequencing technology and reliable detection of mutated genes (110-112).

The existing methods for screening neoantigens involve identification of somatic mutations, HLA typing, HLA-neoantigen affinity prediction and neoantigen immunogenicity validation (Fig. 4).

Identification of somatic mutations. Tumor mutation sites are filtered by performing exome sequencing of tumors and adjacent normal tissue (110,113), aligning the sequence reads

Table I. Commonly used software for somatic mutation analysis.

Software	Function	(Refs.)
Fastp	Quality inspection and quality control	(114,115)
BWA	Sequence alignment	(116)
Bowtie2		(117)
STAR		(118)
HISAT2		(119-121)
TopHat2		(122)
Samtools	Sorting of comparison results	(123)
StringTie	Transcript assembly	(124)
MuTect2	Somatic mutation detection	(125)
VarScan		(126)
VarDict		(127)
Strelka2		(128)
STAR-Fusion	Gene fusion detection	(129)
Annovar	Variant site annotation	(130)
Maftools	Tumor mutation burden calculation	(131)
DESeq2	Gene differential expression analysis	(132)

BWA, burrows-wheeler aligner; STAR, spliced transcripts alignment to a reference; HISAT2, hierarchical indexing for spliced alignment of transcripts 2; Samtools, sequence alignment/map tools; Annovar, annotate variation; Maftools, mutation annotation format tools.

with the reference genome and using mutation identification tools (Table I) (114-132) to detect somatic mutations (such as SNVs and INDELs). RNA-seq can identify neoantigens generated from gene fusions, along with other biological information, such as gene expression levels and copy number variations (133), which can refine the selection of tumor mutation sites. Combining WES data with RNA-seq can confirm whether mutated genes are expressed in tumor cells, thereby filtering more reliable candidate neoantigens.

HLA typing. HLA is a key molecule in the immune system that is involved in antigen recognition. It is encoded by the HLA gene complex located on chromosome 6p21.3 and is primarily involved in functions such as antigen recognition and presentation and immune response regulation, exhibiting a high degree of genetic polymorphism (134). Based on structural and functional differences, HLA is divided into three classes: HLA I, HLA II and HLA III (135). HLA I and II encode molecules that bind and present antigens, allowing cytotoxic T lymphocytes to interact with mature HLA cell surface proteins through antigen-binding grooves and enabling the immune system to recognize and respond to antigens. HLA I genes present endogenous antigens (such as tumor antigens) to $CD8^+$ T cells (136), activating cytotoxic immune responses, and thereby helping the immune system identify and eliminate tumor cells. By contrast, HLA II molecules are primarily responsible for presenting exogenous antigens, serving a key role in initiating immune responses in $CD4^+$ T cells (136), and participating in immune regulation. HLA III genes are located between HLA I and II (135), and functions of these genes remain unclear. In the application of tumor vaccines, HLA typing affects the prediction of neoantigens (137), thereby affecting vaccine preparation. Therefore, accurate HLA typing is key for personalized immunotherapy.

In clinical practice, HLA typing commonly employs three methods: PCR sequence-specific primers and oligonucleotides and sequence-based typing (SBT) (138). Among these, the SBT method has become the gold standard for HLA typing, as recommended by the World Health Organization, because of its high efficiency and accuracy. However, these typing methods are labor-intensive and expensive. Therefore, with the widespread adoption of next-generation sequencing (NGS) technology, second-generation sequencing is gradually being used for HLA gene typing (139). Compared with other methods, NGS technology offers advantages such as high throughput and speed, and the ability to simultaneously detect classical HLA I and II genes, as well as non-classical HLA I genes (140,141). Compared with sequence-specific oligonucleotide probe (SSOP) methods, NGS-based genotyping reduces reagent consumption, minimizes technical variability between experiments, and significantly improves the resolution of HLA allele typing (142). Furthermore, current HLA typing prediction software can achieve up to 99% accuracy in predicting HLA class I alleles (143). Several tools are available for predicting HLA molecular types (Table II) (144-160), among which, OptiType, Polysolver and PHLAT are considered the best performing HLA I typing prediction tools (161,162). HLA typing obtained from WES shows better predictive results than RNA-seq data (149).

HLA-peptide affinity prediction. HLA molecules exhibit high numbers of polymorphisms, with each HLA molecule recognizing a range of peptides and presenting antigenic peptides to T lymphocytes, thereby triggering an immune response. Although the number of short peptides generated by mutations

Table II. HLA typing software

Software	Release date	HLA type	Scope of application	(Refs.)
seq2HLA	2012	I, II	RNA-seq	(144)
HLAminer			RNA-seq, WES, WGS	(145,146)
HLAforest	2013		RNA-seq	(147)
ATHLATES			WES	(148)
OptiType	2014	I	RNA-seq, WES, WGS	(149)
PolySolver	2015		WES	(150)
HLAreporter		I, II	WES, WGS	(151)
HLA-VBSeq			WGS	(152)
xHLA	2017		WES, WGS	(153)
HLA-HD		I	RNA-seq, WES, WGS	(154-156)
HLAscan		I, II	WES, WGS	(157)
ALPHLARD	2018			(158)
PHLAT			RNA-seq, WES	(159)
arcasHLA	2020		RNA-seq	(160)

seq, sequencing; WES, whole exome sequencing; WGS, whole genome sequencing; PHLAT, precise human leukocyte antigen typing; ATHLATES, accurate typing of human leukocyte antigen through exome sequencing; HLA-HD, HLA typing from high-quality dictionary.

is high, tumor antigens are often masked by polysaccharides or other molecules, making it difficult to process and effectively present these antigenic peptides. Ultimately, the number of peptide segments that can bind HLA and be recognized by T cells to trigger an immune response is limited (163). Of 100-200 peptides generated by mutations, only one may bind to a specific HLA molecule (164). Therefore, a large number of candidate false-positive neoantigen peptides exist between the identification of tumor somatic mutations through sequencing and the recognition of neoantigens by TCRs that lead to an immune response.

Traditional screening of candidate positive peptides relies on cytological experiments such as competitive binding (165) and enzyme-linked immunospot (ELISPOT) assays (166). However, depending solely on these experimental methods is often time-consuming and labor-intensive. Therefore, techniques such as genomics and bioinformatics to predict the affinity between HLA and antigen peptides and select suitable tumor antigens are key in immunotherapy (167).

Currently, a number of peptide-HLA binding affinity datasets are available for the training and validation of prediction software, including the Immune Epitope Database (IEDB) (168), SYFPEITHI (169), and MHCBN (170). The development of online prediction algorithms for peptide-MHC affinity is progressing, with the accuracy of predictions improving (171,172). The discovery of allele-specific motifs (173) has enabled algorithms to provide more precise predictions (174). Bioinformatics software tools have emerged to predict the affinity between HLA and antigen peptides; commonly used tools include NetMHC (175), NetMHCpan (176), SMM (177) and SMMPMBEC (178). These prediction tools primarily rely on three types of algorithms: Machine learning (ML), structure-based approaches, and linear regression (LR). ML-based methods predict peptide-binding affinity by learning a function that maps a peptide to regions of binding affinity based on available known binding peptides (179). Representative software includes the artificial neural network-based method NetMHC (175) and the pan-specific method NetMHCpan (176). Structure-based methods predict peptide-binding affinity by calculating the minimum free energy of the pHLA (180) using residue-based statistical energy functions, quantitative structure-activity relationship analysis and quantitative sequence-activity models (181,182). However, owing to the limited number of available crystal structures, the prediction speed and accuracy of these methods are relatively low (183). The LR-based method, a matrix-based approach, predicts peptide-binding affinity by constructing a matrix from the alignment of peptides that represent the motif information (184). As the linear computational complexity is lower than the non-linear computational complexity of other methods, these methods can quickly predict the binding affinity. In addition to these mainstream prediction algorithms, certain software programs integrate multiple methods or use their own methods. For example, TripHLApan (185) combines triple-encoding matrices and recurrent neural networks, ForestMHC (186) uses a random forest classifier and Anthem (187) develops an aggregating one-dependence estimators model based on Bayesian ensemble methods to predict the binding affinity between peptides and HLA (Table III) (169,175-178,184-226).

Prediction algorithms for HLA I molecules are mature, but there are relatively few algorithms for HLA II. Unlike HLA I molecules, the peptide-binding groove of HLA II molecules is open, leading to variations in both the length of peptides that bind to HLA-II and the position of the binding core (227). The most common peptide lengths that bind to HLA II range from 13 to 25 amino acids (228), while class I peptides typically range from 8 to 15 amino acids (195). To the best of our knowledge, there is no database regarding the interactions between HLA II and neoantigens. Therefore, compared with

Table III. Peptide-HLA affinity prediction software.

Software	Release date	HLA type	Algorithm	(Refs.)
SYFPEITHI	1999	I, II	MHC ligands and peptide motifs	(169)
Rankpep	2002		PSSM	(188-190)
NetMHC	2003	I	ANN	(175,191-193)
SMM	2005		SMM	(177)
comblib	2008		Positional scanning combinatorial library	(194)
SMMPMBEC	2009		PMBEC	(178)
NetMHCpan			ANN	(176,195-197)
Pickpocket			PSSM	(184)
MixMHCpred	2017		PWM	(198,199)
PSSMHCpan			PSSM	(200)
MHCflurry	2018		NN	(201,202)
MHCSeqNet	2019		DNN	(203)
ForestMHC			Random forest classifiers	(186)
ACME			CNN	(204)
DeepHLApan			RNN	(205)
HLAthena	2020		CNN	(206)
MHCnuggets		I, II	DNN	(207)
DeepNetBim	2021	I	CNN	(208)
Anthem			AODE	(187)
BigMHC	2023		DNN	(209)
TripHLApan	2024		Triple coding matrix + transfer learning	(185)
TEPITOPE	1999	II	PSSM	(210)
ProPred	2001			(211)
SVRMHC	2006		SVR	(212)
NetMHC II	2007		ANN	(213-215)
NetMHCpan II	2008			(197,215-218)
MHCIIMulti			Multiple instance learning	(219)
MultiRTA	2010		RTA	(220)
TEPITOPEpan	2012		PSSM	(221)
MARIA	2019		DNN	(222)
MixMHC2pred	2019		MoDec	(223,224)
NeonMHC2			CNN	(225)
DeepSeqPan II	2022		DNN	(226)

MARIA, major histocompatibility complex analysis with recurrent integrated architecture; ACME, Attention-based Convolutional neural networks for MHC Epitope binding prediction; PSSM, position-specific scoring matrix; SMM, stabilized matrix method; PMBEC, peptide-major histocompatibility complex binding energy covariance matrix; PWM, position weight matrix; NN,neural network; ANN, artificial neural network; CNN, convolutional neural network; DNN, deep neural network; RNN, recurrent neural network; AODE, average one-dependence estimator; SVR, support vector regression; RTA, regularized thermodynamic average; MoDec, motif deconvolution algorithm; HLA, human leukocyte antigen; MHC, major histocompatibility complex.

the prediction methods for HLA I, the binding predictions for HLA II are in a developmental stage and face more challenges in practical applications (229).

Despite the progress in predictive algorithms, currently available tools cannot reliably predict the immunogenicity of peptides. Several comparative studies have been conducted to evaluate the performance of software packages in predicting affinity (230-234). The results show that artificial neural network algorithms exhibit better predictive performance, with no notable differences between the methods. Concurrently, there are considerable differences in the results predicted by these bioinformatics software programs, depending on the algorithm, MHC type and peptide length (233). Regarding decision thresholds, certain commonly recommended thresholds [half maximal inhibitory concentration (IC50)≤500, percentile rank ≤2]

can lead to low sensitivity, potentially overlooking many MHC ligands (235,236). Additionally, applying a lower binding affinity threshold (IC50≤5,000) generally does not improve prediction performance. The strong binding affinity threshold resulted in the most stable accuracy across different predictors, but also resulted in the lowest accuracy values. Using strong (IC50≤50) and intermediate (IC50≤500) binding affinity thresholds, provides high predictive specificity (233).

To predict pHLA binding affinity more accurately, integrated platforms for prediction methods have emerged (237), such as MHCcombine (233) and IEDB (168). Compared with individual methods, using multiple approaches is more helpful in screening for neoantigens, as algorithms vary in modeling strategies, training datasets, and predictive performance. By combining multiple approaches, the limitations of individual methods can be compensated for, improving both the reliability and accuracy of predictions. This enables a more comprehensive and effective identification of potential neoantigen candidates. However, their predictive accuracy remains unsatisfactory, especially in clinical application (238). Other factors affect the final immunogenicity of the predicted epitopes. These include gene expression, RNA splicing, protein processing and overall patterns of peptide loading and presentation by MHC (239,240). Therefore, the prediction of HLA-peptide binding needs to be improved based on these factors.

5. Evaluation of candidate neoantigen immunogens

Immunogenicity refers to the ability of peptides to bind MHC molecules and induce adaptive immune responses. The key factors in this process are MHC molecule presentation and TCR recognition (241). Not every expressed mutant peptide will be processed by MHC molecules and presented on the cell surface, nor do all pHLAs induce T cell activation and trigger an immune response (242,243). A review of 13 published studies predicting candidate neoantigens as MHC binders demonstrated that out of 1,948 new peptide-MHC combinations, only 53 elicited T cell responses (244). Therefore, it is important to validate the immunogenicity of neoantigens.

Computer-aided prediction. Full activation of T cells requires two signals. The first signal originates from the specific binding of the TCR to pHLA. The second signal originates from costimulatory molecules expressed by APCs that interact with and activate T cells (245). Numerous tools are available for predicting the recognition of neoantigen-specific T cells, which assists in the preliminary screening of the immunogenicity of neoantigens. Commonly used tools such as NetCTL (246) and NetCTLpan (247) generate a comprehensive score by analyzing MHC presentation, proteasomal C terminal cleavage, and transporter associated with antigen processing (TAP) transport efficiency (rather than directly predicting T cell binding) to identify potential cytotoxic T lymphocyte (CTL) epitopes within protein sequences. There are also machine and deep learning techniques to predict TCR and pHLA binding (248). These prediction tools screen neoantigens further, reducing the experimental workload and the consumption of scarce experimental materials.

ELISPOT. Traditional antigen screening methods rely primarily on immunochemical luminescence to detect the strength of T cell effector functions in response to antigen stimulation. When TCRs recognize their target antigens, T cells are activated and subsequently release cytokines such as IL-2 and IFN-γ, as well as granzyme and perforin, leading to the lysis and death of target cells. Numerous detection methods assess the immunogenicity of antigens based on the levels of cytokine release, such as ELISA (249) and ELISPOT (250,251). The most common method is the ELISPOT technique, which evaluates TCR antigen reactivity by detecting changes in the cytokines secreted by T cells in response to antigen stimulation through antibodies (252). This technique is widely used to assess the responses of $CD4^+$ and $CD8^+$ T cells to antigens or mitogenic stimulation (253,254). Owing to its simplicity and high sensitivity, the ELISPOT assay is currently the primary method used for immunological validation.

Chromium release assay. Chromium release assay is used to measure the cytotoxic activity of effector cells *in vitro* (255). This method quantifies the specific killing of target cells by T cells by co-culturing T cells with target cells labeled with the radioactive isotope ^{51}Cr, then detecting the number of radioactive pulses of ^{51}Cr released into the supernatant following lysis of the target cells (256,257). The Cr release assay has the advantages of accurate results and high reproducibility; however, owing to the short half-life of ^{51}Cr, it is difficult to perform multiple measurements. In addition, ^{51}Cr is radioactive, posing health risks to researchers and requiring training in the handling of radioactive materials. These factors limit the applicability of this method.

Organoids. Organoids refer to a technology involving the culture of pluripotent stem cells derived from human tissue in a semi-solid matrix in the presence of mitogens and pathway regulators (258). Compared with traditional cancer cell lines, *in vitro* tumor organoids have greater clinical relevance in tumor immunology: Their heterogeneous three-dimensional structure and spatial arrangement reflect the genomic, morphological and physiological characteristics of the original tumor, making them more likely to accurately predict patient response to drugs (259,260). This provides more precise efficacy and effectiveness data for drug screening, aiding identification of resistance mechanisms or understanding the reasons for treatment failure. Co-culturing tumor organoids with autologous immune cells can demonstrate the cytotoxic ability of T cells against tumor organoids and test the immunogenicity of tumor vaccines (261). Organoids effectively retain the characteristics of the tumors within the patient (262), contributing to the development of personalized treatment plans.

Humanized mouse models. Humanized mouse models have been created by transplanting human tissue (such as the fetal thymus and liver tissue), peripheral blood mononuclear cells (PBMCs), or $CD34^+$ human hematopoietic stem cells and their progenitors (263,264) into immunodeficient mice to simulate the human immune system. Subsequently, human cancer cell line- and patient-derived xenografts are implanted for immune validation experiments (Fig. 5). Among humanized mouse models, PBMC-based models are the most widely

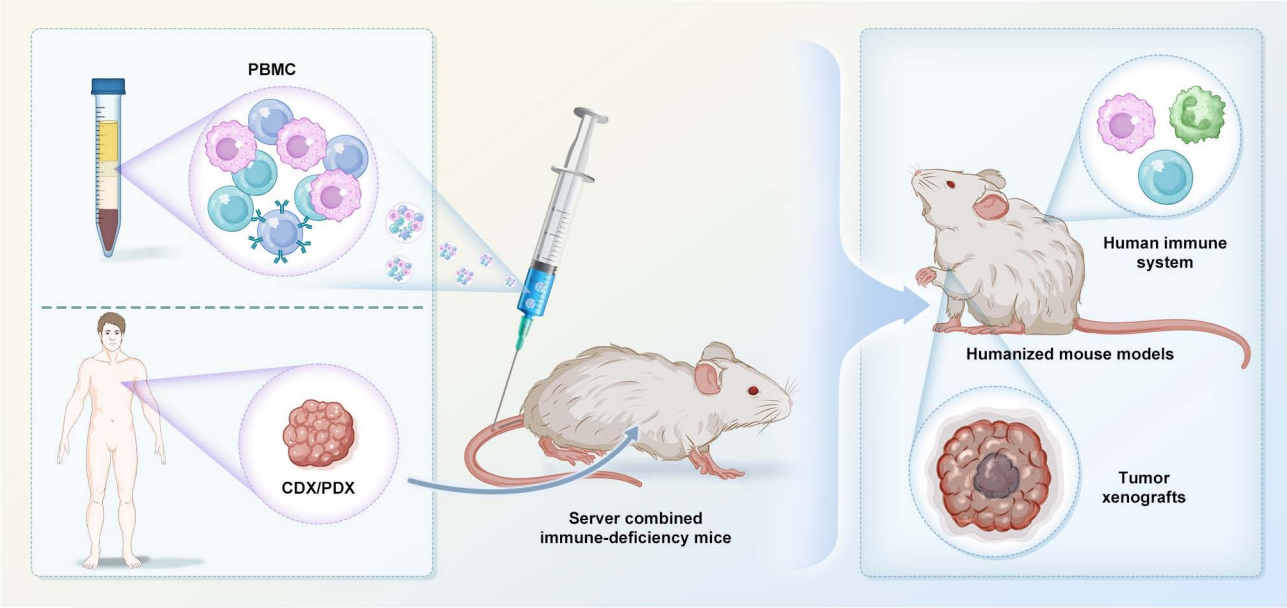

Figure 5. PBMC-humanized mouse tumor model. The PBMC-humanized mouse tumor model is established by transplanting human PBMCs into severe combined immune-deficiency mice, partially reconstructing and simulating the human immune system within the murine host. Human CDXs or PDXs are implanted to generate a tumor model within a humanized immune environment. This model recreates key features of the human immune system, making it a valuable tool for studying the tumor immune microenvironment, evaluating novel immunotherapeutic strategies and identifying potential immunotherapy targets. PBMC, peripheral blood mononuclear cell; CDX, cell-derived xenograft; PDX, patient-derived xenograft.

used due to their relative ease of implementation and lower cost (265,266). These humanized mouse models effectively simulate human immune responses to determine tumor immune evasion mechanisms, test novel immunotherapeutic strategies, and validate the safety and efficacy of drugs (267). Humanized mouse models have achieved notable results in the testing and validation of immunotherapy and are widely used in preclinical research (268,269). Camacho *et al* (270) assessed a carcinoembryonic antigen (CEA) vaccine using transgenic non-obese Diabetic/severe combined immunodeficiency (NOD/scid)-DR1 mice and detected CEA-specific cellular immune responses. In another study, Spranger *et al* (271) developed PBMC-NOD/scid IL2Rg^{null} (NSG) mouse models to evaluate melanoma-associated antigen MART-1 vaccine, and the results showed that the vaccine could induce antigen-specific CD8$^+$ T cell responses. These findings suggest that humanized mouse models effectively recapitulate human immune cell responses to tumor antigens and serve as valuable tools for studying anti-tumor immune responses.

6. Research and clinical applications of neoantigen-based tumor immunotherapy

Melanoma. Melanoma is a highly aggressive and metastatic malignant tumor that is often associated with ultraviolet radiation exposure. Owing to its high tumor mutational burden (TMB) (272,273), melanoma is an ideal target for neoantigen-based immunotherapy (274,275).

In 2015, Carreno *et al* (95) conducted a phase I clinical study of neoantigen DC vaccines for patients with advanced cutaneous melanoma, which demonstrated that the neoantigen DC vaccine enhances spontaneous HLA I-restricted T cell responses, providing experimental evidence for the induction of T cell responses by neoantigen vaccines.

Sahin *et al* (96) reported the first application of an RNA-based polyepitope vaccine for melanoma in humans. All patients exhibited T cell responses and two patients showed notable T cell infiltration into metastatic lesions following vaccination, leading to neoepitope-specific tumor cell killing. Notably, one patient achieved complete remission after receiving the vaccine in combination with the PD-1 blockade, demonstrating the feasibility, safety and antitumor activity of the vaccine in clinical application. In addition, Ott *et al* (58) showed that personalized neoantigen vaccines induce CD8$^+$ T cell responses in 16% of cases and CD4$^+$ T cell responses in 60% of cases; among six patients, four remained recurrence-free 25 months post-vaccination, while the two patients who experienced recurrence achieved complete tumor regression following PD-1 inhibitor treatment. Ott *et al* (276) further evaluated the effects of the personalized neoantigen vaccine NEO-PV-01 in combination with PD-1 blockade in patients with advanced melanoma, NSCLC and bladder cancer: Vaccination effectively induced neoantigen-specific CD4$^+$ and CD8$^+$ T cell responses, with no severe adverse events, further supporting the safety and efficacy of this combination therapy.

Hu *et al* (59) assessed the clinical effects of the NeoVax vaccine in eight patients with surgically resected stage IIIB/C or IVM1a/b melanoma and observed persistent neoantigen-specific T cell responses as well as T cell clonal expansion and diversification. Notably, when combined with ICIs, this approach may be more effective in controlling metastatic tumor progression. Mørk *et al* (277) developed a personalized neoantigen peptide vaccine, EVX-01, which includes a novel CD8$^+$ T cell-inducing adjuvant, CAF®09b, for treating metastatic melanoma. When combined with ICIs, all

five patients exhibited EVX-01-specific CD4+ T cell responses, and the vaccine induced durable neoantigen-specific cellular immune responses. Following combination of EVX-01 immunotherapy and anti-PD-1 therapy, two patients achieved partial response (PR) and one patient achieved complete response (CR). All immune-related adverse effects were grade 1, further demonstrating the potential of personalized neoantigen vaccines in combination with ICIs.

In the KEYNOTE-942 trial (278), the efficacy of a personalized neoantigen vaccine, mRNA-4157, in combination with pembrolizumab for advanced melanoma was evaluated. The results showed that this combination therapy significantly prolonged recurrence-free survival (RFS) and decreased recurrence and mortality rates, providing new research directions for adjuvant selection and combination of multiple immunotherapeutic approaches.

Lung cancer. Lung cancer is the most common cancer worldwide, accounting for 12.4% of newly diagnosed cases (1). The application of neoantigen vaccines for the treatment of lung cancer has also progressed. Phase I/II clinical trials are currently evaluating the safety and immunogenicity of neoantigen vaccines (279-284) and their role as adjuvant interventions for other immunotherapies.

Ding *et al* (279) treated 12 patients with metastatic lung cancer using a DC vaccine based on neoantigens. The disease control rate was 75%, with a progression-free survival (PFS) of 5.5 months and an overall survival (OS) of 7.9 months. All treatment-related adverse events were grade 1-2, indicating that this therapeutic approach has a favorable safety profile.

Recent studies on tumor neoantigen vaccines have demonstrated promising efficacy in the treatment of NSCLC (280,281). Li *et al* (282) reported the results of a phase I clinical trial involving 24 patients with stage III/IV NSCLC who received personalized neoantigen peptide vaccines. Following vaccination, the median PFS was 6.0 and the median OS was 8.9 months. Additionally, all patients exhibited a significant increase in the frequency of neoantigen-specific CD8+ T cells in peripheral blood following the vaccination process. In the KEYNOTE-603 study (280), 16 patients received treatment with the mRNA-4157 vaccine, including 11 patients with NSCLC; of these patients, 14 remained disease-free throughout the study. This vaccine has progressed to a phase III clinical trial (281).

The efficacy of neoantigen vaccines in combination with other treatment modalities for lung cancer is widely recognized (283,284). As demonstrated by Ott *et al* (276), the combination of neoantigen vaccines with ICIs can enhance the immune system antitumor response. In a case report (283), a patient with advanced squamous cell carcinoma who was resistant to PD-1 blockade was treated with a neoantigen DC vaccine combined with ICIs. The results showed significant tumor regression, with OS of 48 months and no severe adverse reactions. Additionally, in a phase Ib clinical trial, Awad *et al* (284) used the personalized neoantigen vaccine NEO-PV-01 in combination with pemetrexed, carboplatin and pembrolizumab as first-line treatment for advanced NSCLC. Following vaccination, neoantigen-specific CD4+ and CD8+ T cell responses were observed along with increased infiltration of CD4+ T cells, which exhibited a pronounced cytotoxic phenotype. These findings validate the potential efficacy of combining neoantigen vaccines with ICIs.

McCann *et al* (285) explored the feasibility of personalized neoantigen vaccines in patients with lung cancer and low TMB using a mouse model. In patients with an unfavorable tumor microenvironment, it was possible to identify antigens capable of effectively activating neoantigen-specific T cells (285). This provides new insights and evidence for personalized immunotherapy in lung cancer with low TMB.

Glioma. Gliomas are the most common primary intracranial tumors of the brain, with a poor prognosis and limited curative treatment options (286). Gliomas are considered immune-privileged tumors (287), leading to limited effectiveness of immunotherapy. However, an increasing number of immunotherapeutic strategies have been developed (288).

Isocitrate dehydrogenase 1 (IDH1) (289,290) mutation-associated vaccines are currently the most common neoantigen vaccines for gliomas (99,291-294), particularly the IDH1 arginine-to-histidine substitution at codon 132 (IDH1 R132H) mutation, which is widely present in grade II and III gliomas as well as 80% of secondary glioblastomas (295). Schumacher *et al* (99) confirmed that IDH1 R132H contains immunogenic epitopes suitable for mutation-specific vaccination. This mutated peptide is presented by MHC class II molecules and induces a mutation-specific CD4+ T-helper-1 immune response (99). Additionally, the study employed an MHC-syngeneic humanized mouse model to administer the IDH1 R132H-specific peptide vaccine. The results demonstrated that the vaccine effectively induced a specific anti-tumor T cell immune response and enhanced anti-tumor efficacy (99). Pellegatta *et al* (292) established a glioma xenograft mouse model carrying the IDH1 R132H mutation and immunized mice with five peptide vaccines containing the mutation. The results showed a significant increase in peripheral CD8+ T cells following immunization, accompanied by elevated IFN-γ levels and the generation of anti-IDH1 antibodies, indicating that the vaccine effectively activated an antitumor immune response.

Platten *et al* (293,294) developed a tumor vaccine targeting IDH1 mutations and conducted a phase I clinical trial in humans. Following vaccination with IDH1-specific peptides, vaccine-induced immune responses involving multiple MHC alleles were observed in 93.3% of participants. Furthermore, the vaccine demonstrated promising clinical efficacy, with 3-year PFS and OS rates of 0.63 and 0.84, respectively. Among patients with a positive immune response, the 2-year PFS rate was 0.82. Moreover, related adverse events were classified as grade 1. The IDH1 R132H mutation-specific vaccine has demonstrated good immunogenicity and safety in preclinical and clinical studies (99,292,293,294).

Due to the notable intratumoral heterogeneity of gliomas, research on glioma neoantigen vaccines has primarily focused on developing personalized vaccines: For example, Hilf *et al* (296) conducted a phase I clinical trial of the Glioma Actively Personalized Vaccine Consortium, in which researchers designed personalized vaccines based on transcriptomic and immunopeptidomic mutation analyses of tumors. The results showed that most patients responded to at least one immunopeptide, with a significant increase in CD8+ T cell

numbers and a transition to a memory phenotype. Additionally, patients exhibited new epitope-specific responses, primarily TH1-type $CD4^+$ T cell responses, while maintaining a good safety profile. In a phase I/Ib study, Keskin *et al* (60) adopted a similar strategy using multi-epitope personalized neoantigen vaccines to treat patients with glioblastoma: Following vaccination, patients developed multifunctional $CD4^+$ and $CD8^+$ T-cell responses to neoantigens.

A phase III clinical trial (297) evaluated the efficacy of a personalized peptide vaccine in HLA-A24-positive patients with recurrent glioblastoma. Although the trial showed OS of vaccinated patients was 8.4 months, which was slightly higher than the 8.0 months in the placebo group, the difference in OS and PFS between the two groups was not significant. Thus, although personalized peptide vaccines exhibit immunostimulatory potential, their clinical benefits require further validation. These studies indicate that the design and application of personalized neoantigen vaccines holds promise for the treatment of gliomas.

Hepatocellular carcinoma (HCC). HCC has a high recurrence rate (298-301). Therefore, studies on neoantigens in HCC have primarily focused on anti-recurrence treatments (302-304). Liu *et al* (302) used neoantigen-reactive T cell-based immunotherapy combined with tomotherapy to treat a patient with advanced HCC. The patient achieved long-term PFS following treatment, demonstrating the potential of the combined therapeutic approach in the treatment of liver cancer. Cai *et al* (303) designed a personalized neoantigen vaccine for 10 patients with resectable HCC and portal vein branch invasion; RFS was prolonged after vaccination, and five patients who exhibited a strong neoantigen response after full vaccination had longer RFS than the others.

Peng *et al* (304) conducted a phase II clinical trial in which a personalized neoantigen DC vaccine was combined with neoantigen-activated T cell therapy to treat 10 patients with HCC who underwent curative resection or radiofrequency ablation. The results showed that 70% of the patients developed a neoantigen-specific T cell response, and 71.4% remained recurrence-free within 2 years of treatment. All adverse events were grade 1 or 2. Shen *et al* (305) treated patients with unresectable HCC patients with radiotherapy combined with an immune therapy composed of personalized peptide vaccination (PPV) peptides, DC, and CTL (PPV-DC-CTL). After radiotherapy and 1-3 cycles of PPV-DC-CTL treatment, the Alpha-fetoprotein (AFP) levels in six patients significantly decreased. Imaging assessments showed PR in three patients, while the other three patients had stable disease. The response rate was 33%, and the disease control rate was 66%. The study found that the regimen was safe and well-tolerated. The aforementioned studies indicate that combining neoantigen-based immunotherapy with other treatments is safe and feasible, and it may help reduce recurrence of HCC after curative treatment, or effectively treat HCC.

Gastric cancer. Guo *et al* (306) designed a neoantigen-based DC vaccine, Neo-MoDC, for treating metastatic gastric cancer. Clinical trial results showed that patients who received the neo-MoDC vaccine alone generate a neoantigen-specific T cell response. When combined with ICIs, a stronger immune response is triggered, leading to complete regression of all tumors within 25 months (306).

Yu *et al* (307) conducted a personalized neoantigen vaccine study on six patients with microsatellite-stable colorectal cancer who experienced recurrence or metastasis following surgery and chemotherapy. Neoantigen-specific immune responses were observed in 66.67% of the vaccinated patients, and four patients remained progression-free until the completion of the clinical trial.

Other types of cancer. Compared with cancers such as melanoma and lung cancer, the development of neoantigen-based therapies for other tumor types is in its early stages. The personalized neoantigen vaccine NEO-PV-01, developed by Ott *et al* (276), has also made breakthrough progress in the treatment of bladder cancer. After synthesizing the antigen peptides, they were mixed with the adjuvant polyinosinic-polycytidylic acid stabilized with poly-L-lysine and carboxymethylcellulose (poly-ICLC) (308) for bladder cancer therapy, demonstrating the vaccine's safety and efficacy (276). Zeng *et al* (309) treated a patient with advanced collecting duct carcinoma (CDC) using a personalized neoantigen vaccine combined with NRTs. Following treatment, both the primary CDC tumor and metastatic lesions remained in a stable disease state for 9 months, suggesting that neoantigen-based immunotherapy may improve the PFS of patients with advanced CDC.

Chen *et al* (310) developed a personalized neoantigen vaccine containing 20 neoantigen peptides for seven patients with advanced pancreatic cancer. The median and vaccine-associated OS and PFS rates were 24.1, 8.3 and 3.1 months, respectively. Notably, one patient had a vaccine-related OS of 21 months, and the abundance of antigen-specific TCR clones increased from 0 to nearly 100%, demonstrating the potential of neoantigen vaccines to activate specific T cell subsets and eliminate cancer cells.

Rojas *et al* (54) treated 16 patients with surgically resected pancreatic ductal adenocarcinoma using a personalized mRNA neoantigen vaccine in combination with multiple therapies. The results showed that at least one neoantigen-specific $CD8^+$ T cell response was induced in half of the patients, and responders had significantly prolonged median RFS. Based on these promising results, a global randomized clinical trial will be conducted to validate the efficacy of this therapy.

Recent studies have identified neoantigens derived from RNA splicing aberrations in numerous types of cancer (93,311-313). These previously uncharacterized shared neoantigens are widely expressed in tumors such as gliomas, mesothelioma and prostate and liver cancer (314). It is hypothesized that these neoantigens are endogenously generated and presented by tumor cells under physiological conditions, effectively triggering neoantigen-specific $CD8^+$ T cell responses to eradicate cancer cells (313,315). This provides a molecular foundation for addressing tumor heterogeneity in immunotherapy.

7. Challenges and future prospects of neoantigen vaccines

Research on tumor immunotherapy has a history of nearly 20 years, with thousands of related clinical research and development projects registered on ClinicalTrials.gov in the United

States. However, the development of tumor vaccines is a long and challenging process. Although the rapid development of bioinformatics and high-throughput sequencing technologies has made the screening of neoantigens more efficient and convenient, with the emergence of more specialized algorithms and tools and both the accuracy and speed of predictions have improved, tumor vaccines based on neoantigens face issues that need to be addressed.

SNVs and INDELs are important neoantigen sources. However, in addition to these, there are other types of neoantigen sources, especially non-coding region neoantigens that have been previously overlooked, which may have higher immunogenicity (107,108,316). Research in this field is in its early stages (317), and relevant screening processes and standards require further optimization and improvement (318).

The prediction and screening of neoantigens is complex because of the variety of mutations involved, the diversity of HLA molecules and the intricate mechanisms of immune presentation (319). Consequently, existing tools and algorithms struggle to simulate and predict the binding and presentation processes of antigen peptides to HLA molecules with 100% accuracy. Therefore, there is room for improvement in the optimization of tools and processes, especially for the prediction of HLA II molecule binding. Most current prediction tools focus primarily on HLA I molecules, whereas the support for HLA II molecules is lacking (229). Enhancing the accuracy of binding predictions and expanding the support for different HLA types are key issues to be addressed (320). Additionally, neoantigen data remains relatively limited. Because neoantigen prediction relies on a number of unknown factors, particularly individual differences and the diversity of the tumor microenvironment (321), the uncertainty of the prediction results is high, which impedes clinical application.

Current research mainly focuses on binding studies between MHC and antigen peptide segments, but there is relatively little research on the role of peptide segments in T cell activation or their therapeutic potential (50,322,323). Although prediction models allow the identification of a large number of neoantigens in clinical trials of cancer vaccines, a small fraction of the predicted antigens exhibit immunogenicity in clinical settings (43,296,324). Relying solely on computational prediction of neoantigens may not accurately reflect their therapeutic effectiveness in clinical application.

Based on the current clinical practice, numerous neoantigen vaccine strategies have been developed using a single biopsy sample (309,310). However, this approach has several limitations. First, tumor heterogeneity means that a single biopsy sample cannot fully represent variations within a tumor. Candidate neoantigens may differ between tumor lesions, metastatic sites and between primary and metastatic tumors (325). Neoantigen vaccines derived from these samples may target a small subset of tumor antigens, thereby limiting its widespread use.

Antigens that provoke a strong tumor rejection response often exhibit individual specificity (326). Only a small number of neoantigens are shared between patients, making it difficult to develop neoantigen vaccines with broad applicability. Most studies have concentrated on personalized tumor vaccines (52,54,133,276-285,297,303,307); however, the development of personalized treatments is time-consuming and costly. In patients with a short therapeutic window, the time is often insufficient to complete the process. Therefore, although personalized tumor vaccines have theoretical potential, developing broadly applicable treatments within a short time remains a challenge.

Although tumor vaccines based on neoantigens have made progress, their efficacy remains suboptimal because of tumor heterogeneity and individual patient differences (325,326). To address these challenges, several strategies have been proposed. Development of novel multi-target antigen vaccines by targeting multiple neoantigens simultaneously can activate a broader immune response, thereby enhancing therapeutic efficacy (50,54,91). Compared with single-neoantigen vaccines, multi-target vaccines may overcome tumor heterogeneity and immune evasion. Current research primarily focuses on tumor-specific neoantigens derived from mutations (97,319). However, recent studies have identified non-traditional sources of neoantigens (98), such as gene fusion and RNA splicing (311-313), which may exhibit high immunogenicity. Additionally, neoantigens generated from aberrant translation in non-coding regions warrant further investigation (107,108,316). In-depth studies of these neoantigens may reveal more effective immune epitopes. Compared with personalized neoantigens, developing shared neoantigens is key for large-scale applications (327). Shared neoantigens can be used across a broader patient population, decreasing research and development costs and shortening treatment timelines, thereby creating favorable conditions for commercial applications. The delivery method greatly influences the efficacy of neoantigen vaccines. Each format (peptides, RNA, DNA, viral vectors, DCs) has pros and cons, and should be chosen based on clinical context and antigen features (171). To enhance therapeutic outcomes, it's essential to establish a comprehensive evaluation system, monitor immune responses, and optimize formulations, adjuvants, and dosages using preclinical models. Exploring optimal combinations of neoantigen vaccines with adjuvants, such as poly-ICLC (328), has demonstrated potential in clinical trials of personalized neoantigen vaccines (24,97,276). Additionally, studies on immune induction mechanisms, including the molecular mimicry of tumor antigens and the ability to initiate antigen epitope spreading, should be conducted to develop more effective vaccine adjuvants and enhance vaccine efficacy. Based on neoantigen vaccine therapy, combining strategies that protect and activate T cells can decrease T lymphocyte apoptosis in patients, thereby ensuring therapeutic efficacy. Additionally, the integration of conventional treatments, radiotherapy, targeted therapy and immunotherapy should be explored to enhance T cell responses to vaccines and improve clinical outcomes (78).

In summary, future research should focus on screening and validation of neoantigens, as well as overcoming the technical difficulties and costs encountered during the clinical translation process (329).

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Authors' contributions

YTJ, BW and HF conceived and design of the study. HF and BW prepared the original draft of the manuscript. HF drew the figures. YTJ and BW revised the manuscript. Data authentication is not applicable. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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