THE CLINICAL APPLICATION OF NEOANTIGENS

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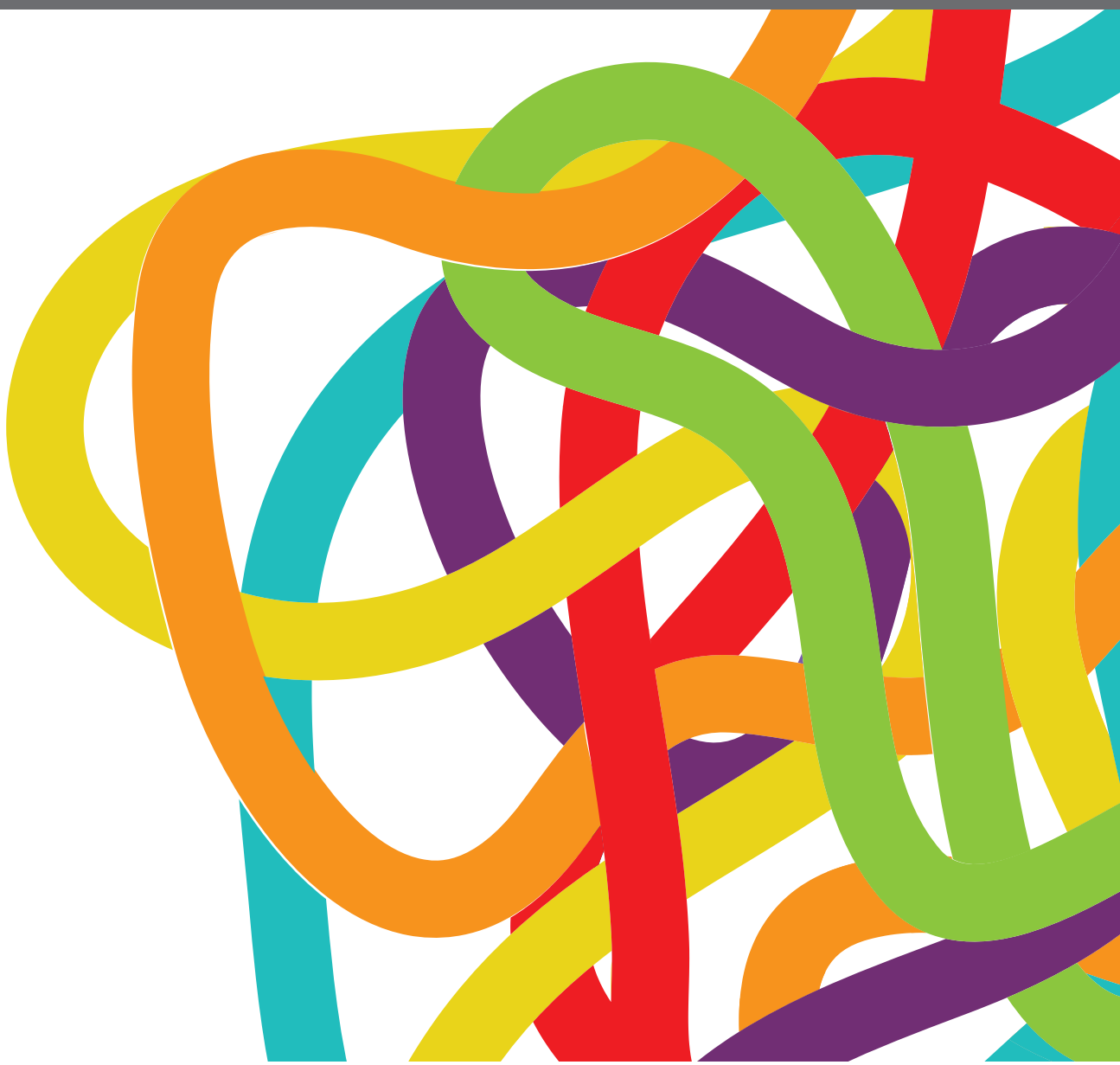

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THE CLINICAL APPLICATION OF NEOANTIGENS

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EDITORIAL
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Editorial: The Clinical Application of Neoantigens

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Editorial on the Research Topic

The Clinical Application of Neoantigens

To a large extent, the specificity of cancer immunotherapy is dependent on the recognition of specific tumor antigens, especially neoantigens. Neoantigens are newly formed antigens that have not been previously recognized by the immune system. Neoantigens can arise from altered tumor proteins formed as a result of tumor mutations or from viral proteins. They are highly restricted to tumor cells, with minimally established immune tolerance. Neoantigens used in vaccines and other types of immunotherapies are being studied in the treatment of many types of cancer. Furthermore, a growing body of evidence indicates that neoantigen-specific T cells underlie the success of the recent surge in immune checkpoint blockades (ICB). Although the origin of neoantigens has been discussed extensively in published literature, the identification of neoantigens and their influence in clinical practice are largely ignored.

The application of neoantigens is rapidly becoming more widespread in clinical settings, not only just related to the development of tumor vaccine or adoptive cell therapy but also in the monitoring of clinical response in ICB and other therapies as well. The present Research Topic titled *"The Clinical Application of Neoantigens"* features 10 articles that reflect the clinical application of neoantigens and develop novel strategies for cancer therapy. This Research Topic includes mostly review articles about neoantigen-identified tools or methods, neoantigens as a basis for immunotherapies, and work using The Cancer Genome Atlas (TCGA), Gene Expression Omnibus (GEO) or own researchers collected datasets to identify novel biomarkers.

Hao et al. proposed a deep convolutional neural network named APPM (antigen presentation prediction model) to predict antigen presentation in the context of human leukocyte antigen (HLA) class I alleles. APPM is trained on large mass spectrometry (MS) HLA-peptides datasets and evaluated with an independent MS benchmark. Finally, they identified 16,000 putative neoantigens with the hallmarks of 'drivers'. Generally, this study is only based on MS datasets, however, Next-Generation Sequencing (NGS) including RNA-sequencing (RNA-seq), whole-genome sequencing (WGS), and whole-exome sequencing (WES) is more frequently used for neoantigens. One has to additionally take into consideration already previously published work that proposed five major types of neoantigen with NGS, such alternative mRNA splicing (AS), chimeric RNAs (or fusion transcripts), circular RNAs (circRNAs), RNA editing, transposable elements (TEs), and human

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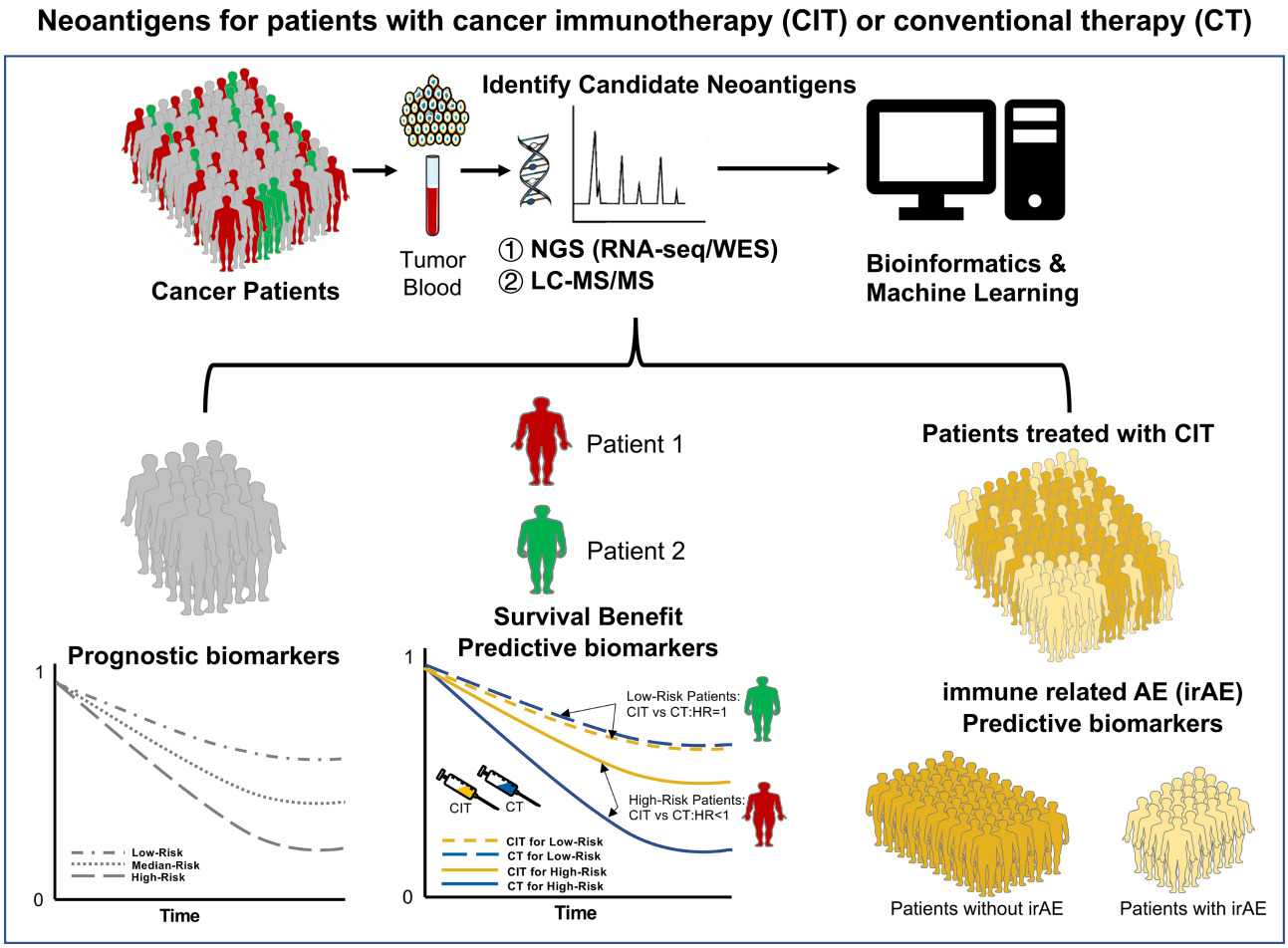

FIGURE 1 | Neoantigens as potential biomarkers for cancer patients treated with immunotherapies and/or conventional therapies. Neoantigens are mostly derived from mutations in tumor cells. The candidate neoantigen can be identified from tumor tissue and blood samples with bioinformatics analysis ①. Next-Generation Sequencing (NGS), including RNA-sequencing (RNA-seq), whole-genome sequencing (WGS), and whole-exome sequencing (WES); ②. Liquid chromatography-mass spectrometry (LC-MS). They can serve as three major potential biomarkers. First, as a prognostic biomarker which indicates an increased (or decreased) likelihood of a future clinical event, disease recurrence, or progression in an identified population. Predictive biomarkers are used to identify individuals who are more likely than similar individuals without the biomarker to experience a favorable or unfavorable effect from exposure to a medical product or an environmental agent. Safety biomarkers are measured before or after an exposure to a medical product or an environmental agent to indicate the likelihood, presence, or extent of toxicity as an adverse effect. Figure modified with text, markings (stars), and annotation after adapted from Servier Medical Art by Servier, licensed under a Creative Commons Attribution 3.0 Unported License. Original photo adapted from https://smart.servier.com/smart_image.

endogenous retroviruses (HERV) (1). Generally, numerous work about various bioinformatics pipelines and tools in this regard have been performed. Prof. Griffith, e.g., already outlined each step in the neoantigen workflow to predict high-quality immunogenic neoantigens (2).

Six review articles in the special issue focus on neoantigens as a basis for immunotherapies. Hereby, Dr. Zhong (Zhang et al.) summarized the latest advances in the classification of immunotherapy and the process of classification, identification, and synthesis of tumor-specific neoantigens, as well as their role in current cancer immunotherapy. Furthermore, prospects and existing problems of neoantigens are discussed, as is the long development cycle of neoantigen vaccine that is recognized as a primary obstacle to the application of vaccine, and the challenge of preparation and delivery of vaccines. Yu et al. introduced pyroptosis and ferroptosis as recently discovered types of programmed cell death (PCD) that are different from apoptosis, necrosis, and autophagy. They highlight that tumor cell neoantigens target tumor cells and cause pyroptosis or ferroptosis which might be an additional strategy for the future. Arnaud et al. provided an overview of the main strategies for T cell receptor (TCR)-engineering, described the

selection and expansion of optimal carrier cells for TCR-adoptive T cell transfer (ACT) and discussed the next-generation methods for rapid identification of relevant TCR candidates for gene transfer therapy. Particularly CRISPR-Cas9 technology was recommended to verify TCR candidates for clinical practice. Zhu and Liu outlined the challenges of targeting neoantigens for cancer treatment. It is warranted to explore the combinatorial approaches with other immunotherapies, including checkpoint blockade therapies or conventional treatments, including chemoradiotherapies, kinase inhibitors, and anti-angiogenesis therapies. Gu et al. provided a clear picture of the clinical application of neoantigens in esophageal cancer (EC) from tumor-specific cancer vaccines and adoptive T cells to combination therapies. Liao and Zhang reported about neoantigen vaccines that were identified in preclinical and clinical trials and summarized the safety and efficacy of personalized cancer vaccines combined with ICBs in several cancer types. Most of the clinical trials are phase I or IB; only three phase II studies are recruiting.

Tumor antigens may be recognized by the immune system as non-self and elicit an immune response. Tumors with high TMB and/or MSI-H/dMMR may lead to an increase in neoantigens. Wang et al. therefore reviewed the potential application of tumor neoantigen burden (TNB) as a biomarker. The impact of high TNB and increased number of infiltrating immune cells on the efficacy of immunotherapies is discussed. Zou et al. summarized neoantigen load (NAL) which is similar to TNB, as a biomarker for predicting the anti-tumor effects of ICB. When NAL alone is insufficient to predict efficacy, its combination with other indicators can improve prediction efficiency. Liu et al. found that overexpression of *ERO1L* was associated with poor prognoses in patients with Lung Adenocarcinoma (LUAD). Overexpression of *ERO1L* was indicative of a hypoxia-induced immune-suppressive TIME, which was shown to confer resistance to immunotherapy in patients with LUAD.

In summary, this Research Topic highlights that tumor neoantigens play an essential role in antitumor immunity and successful cancer immunotherapies regardless of cancer vaccine alone or combinations with ICB. With the development of bioinformatics pipelines and tools, more novel neoantigens including circRNA, AS, fusion, and TE, are identified. Thereby, neoantigen-based tailored therapies can be widely performed in various cancers soon. Neoantigens could serve as potential biomarkers for cancer patients treated with immunotherapies. In the next decades, we still need to investigate more and more novel neoantigens as potential prognostic, predictive, and safety biomarkers for cancer patients treated with immunotherapies alone and/or conventional therapies such as RT and CT (**Figure 1**). Let's go ahead with translational research of neoantigens for cancer!

AUTHORS CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Beyond Tumor Mutation Burden: Tumor Neoantigen Burden as a Biomarker for Immunotherapy and Other Types of Therapy

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Immunotherapy has significantly improved the clinical outcome of patients with cancer. However, the immune response rate varies greatly, possibly due to lack of effective biomarkers that can be used to distinguish responders from non-responders. Recently, clinical studies have associated high tumor neoantigen burden (TNB) with improved outcomes in patients treated with immunotherapy. Therefore, TNB has emerged as a biomarker for immunotherapy and other types of therapy. In the present review, the potential application of TNB as a biomarker was evaluated. The methods of neoantigen prediction were summarized and the mechanisms involved in TNB were investigated. The impact of high TNB and increased number of infiltrating immune cells on the efficacy of immunotherapy was also addressed. Finally, the future challenges of TNB were discussed.

Keywords: tumor neoantigen burden, biomarker, immunotherapy, immune response, tumor mutation burden

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INTRODUCTION

Tumor immunotherapy aims to control tumor development by activating the immune system to attack tumor cells. By selecting appropriate antigens, notably neoantigens produced by tumor-specific mutations, an effective tumor-specific immune response can be mounted, and immune tolerance can be minimized (1). Non-synonymous somatic mutations will produce altered peptides, among which, some are processed and presented by the major histocompatibility complex (MHC) in order to generate neoantigens. These molecules are the key factors required for successful immunotherapy, including immune checkpoint inhibitors (ICIs), personalized tumor vaccines and adoptive T cell transfer immunotherapy (2–4). These strategies have shown promise in the treatment of solid tumors (5–7).

A higher number of DNA mutations are associated with higher number of candidate peptides, and results in an increased probability of successfully presented neoantigens (8). The response to immunotherapy correlates with tumor mutation burden (TMB) and mainly with the number of mutations in the coding region of the genome (exome) of the tumor cells. It is usually reported as the number of mutations present in a megabase of the genomic region by whole-exome sequencing or large-scale next-generation sequencing (9–12). Similarly, the tumor neoantigen burden (TNB) is defined by the number of neoantigens per megabase in the genome region (13, 14). Notably, TMB

has become a biomarker for immunotherapy, assuming that higher TMB will increase the probability of tumor neoantigens and specific T-cell responses (15).

However, the role of TMB in immunotherapy remains controversial (16–18), since not all mutations produce neoantigens. Only a limited number of mutations can be properly processed, presented on the surface of the MHC complex and recognized by T cells (19). The TMB noted in pediatric tumors is considerably low (20). However, in certain tumors, such as pediatric medulloblastoma or acute lymphoblastic leukemia, which exhibit minimal mutational burden, a strong anti-tumor immune response can be induced by high-quality neoantigens (21, 22).

TMB generates neoantigens and causes tumor immunogenicity. This biomarker can be used as a valuable estimate of TNB to a certain extent. A positive correlation has been noted between TMB and TNB. However, TNB is directly used for neoantigen evaluation and may be considered an improved biomarker for immunotherapy compared with TMB (23–25). High TNB was associated with durable progression-free survival (PFS) in patients with non-small cell lung cancer (NSCLC) treated with programmed death 1 (PD-1) inhibitors (26). In addition, TNB correlated with clinical benefit in patients with metastatic melanoma treated with cytotoxic T-lymphocyte-associated protein 4 (CTLA4) inhibitors (27). Similarly, a phase I/II trial performed in patients with stage IV melanoma demonstrated that their clinical benefit was associated with a proposed immune activation signatures score. Among the score items, high TMB and predicted TNB were significantly associated with improved PFS and overall survival (28). The present review investigated the application of TNB as a biomarker in immunotherapy and other therapies and provided an in-depth discussion of the mechanisms, clinical application and challenges of this biomarker.

NEOANTIGEN PREDICTION

In general, *in silico* analysis on genome sequencing can aid the selection of immunogenic neoantigen peptides. Neoantigen prediction is usually performed prior to selecting immunogenic neoantigens to reduce the burden of immunogenicity testing by decreasing the number of candidate peptides. This is a necessary step in developing personalized immunotherapy. Several important steps are involved in neoantigen selection, including intracellular processing and transportation, the stability and affinity of peptide-MHC complex binding, the diversity of T cell receptors (TCR) and the recognition by TCR. In addition, the difference between the prediction algorithms is also important. Neopepsee is a neoantigen prediction algorithm that automatically extracts mutated peptide sequences and expression levels, and combines multiple immunogenic features to construct a machine-learning classifier (29). The application of deep learning to the determination of large human leukocyte antigen (HLA) peptides and genomic data sets from various tumors can aid the development of a computational model for neoantigen prediction (30). An additional prediction algorithm can determine the priority of neoantigens and discover immune characteristics in cancer immunotherapy by the classification of human neoantigen/neopeptide data into three categories based on different mutation positions (anchor mutation, MHC-contacting position and TCR-contacting position) (31).

Several computational pipelines have been developed for neoantigen prediction. However, the majority of them are based on peptide affinity with MHC (32–34). Furthermore, neoantigen prediction can be performed by prioritizing predicted peptides based on mutant allele expression, mutation clonality, MHC presentation, and T cell recognition, either alone or in combination (35–38). A Cauchy-Schwarz index of neoantigens score was proposed and the effects of both clonality and MHC binding affinity were included in order to accurately determine the concentration of neoantigens in truncal mutations (39). An additional prediction model was developed by integrating peptide presentation and recognition into antigenic determinant immunogenicity *via* the use of specific parameters.

To establish a global neoantigen prediction algorithm standard, several institutions established the tumor epitope selection alliance, which is a bioinformatics consortium with scientists from well-respected neoantigen research groups. These institutions independently mine the open database of tumor sequencing, predict potential neoantigens and rank candidate peptides. Different predictions may be collected and cross-matched to reach a final optimized consensus. This integration incorporates aspects of binding affinity, tumor abundance, stability and peptide identification in addition to antigen presentation. Therefore, higher precision would be expected (40).

THE MECHANISMS OF TNB FORMATION

Any form of genomic instability, including single -nucleotide variation (SNV), frameshift mutations, splicing variations or chromosome rearrangement, may result in TNB. The genomic instability can result from abnormalities in either DNA replication or mismatch repair (MMR) (41). The high-fidelity process of DNA replication requires replicative DNA polymerases, exonucleolytic proofreading and MMR. Abnormalities that may occur in any of these parts contribute to genetic instability. Inactivation of DNA polymerase leads to excessive mutations, such as ultra-hypermutated phenotype. Defective MMR (dMMR) leads to microsatellite instability (MSI), which is an ultra-hypervariable phenotype of short repetitive DNA sequences and SNV. Following exposure to either exogenous (smoking, ultraviolet radiation, chemicals, ionizing radiation) or endogenous (reactive oxygen species, endocrine abnormalities) mutagens, dMMR/MSI facilitates carcinogenicity and paradoxically increases TNB, which in turn enhances immunogenicity (42–46).

Although TNB is a biomarker of immunotherapy, current knowledge regarding its function is limited. Primarily, TNB analysis was performed on SNV (47). However, other genetic

aberrations may produce comparable or even more immunogenic neoantigens (23). For example, neoantigens were found from a data set of gene fusion-positive tumors (48). Splice variants are also sources of neoantigens. High expression of PD-1 and programmed death-ligand 1 (PD-L1) were observed in tumors with splice variants (49). In addition, a new class of neoantigens was discovered, which was derived from intra- or inter-chromosomal rearrangements (50).

Various studies have analyzed the frequency of specific somatic mutations in multiple types of cancers, which demonstrated that the frequency of non-synonymous mutations varied greatly, ranging from ~0.001/Mb to higher than 400/Mb. The mutation frequency was very prominent in melanoma and lung cancer (20, 51). Notably, Samra Turajlic et al. analyzed and compared the counts of Insertion-and-deletion-derived tumor-specific neoantigens in pan-cancer, both SNV-derived neoantigens and frameshift indel-derived neoantigens in the study showed that melanoma, lung cancer, bladder cancer, colon cancer, and head and neck cancer with high TNB (47). Mutations in lung cancers can be attributed to direct DNA damage from cigarette smoke carcinogens. A significant dose-response association of smoking history with genetic alterations has been noted in advanced non-small-cell lung adenocarcinoma with regards to cancer-associated pathways and their corresponding mutant antigens (52, 53). Ultraviolet stimulation is the main factor leading to high TNB in melanoma (54). In other common cancer types, such as colorectal or endometrial cancer, which harbor DNA polymerase epsilon mutations, increased TNB is attributed to endogenous mutations (55, 56). However, in certain tumors, such as bladder cancer, the mechanisms underlying the formation of TNB are complex, including the apolipoprotein B mRNA editing enzyme catalytic polypeptide family, smoking, viral infection and genetic fusions (57).

A previous study dissected the genetic heterogeneity during the evolution of a primary osteosarcoma tumor to its metastatic variant. Metastases exhibited higher TNB compared with primary tumors, possibly due to the accumulating mutations in DNA damage response genes (58). Different mutational landscapes exist between the primary and metastatic sites and in the subclones noted inside different regions of a tumor (intra-tumoral heterogeneity; ITH). The presence of ITH suggests that the tumor cannot elicit equal immunity. Patients with tumors exhibiting high TNB and low ITH are more likely to benefit from immunotherapy (59, 60).

TNB CORRELATES WITH TUMOR-INFILTRATING LYMPHOCYTES

Neoantigens alone are not sufficient to mount an effective immune response and tumor-infiltrating lymphocytes (TILs) are also required for this process (61, 62). High TNB can promote the recognition and activation of T cells, which in turn increase TILs and improve the immune response of cancer patients to cancer cells (**Figure 1**). Colorectal tumors with dMMR exhibit neoantigen-stimulated lymphocyte infiltration and increased levels of inflammatory cytokines. In the absence of high TMB, high TNB alone correlates with the inflammatory microenvironment (63). Moreover, lung adenocarcinoma patients with high TMB presented with enhanced infiltration of activated $CD4^+$ and $CD8^+$ T cells, while the mutations detected could accurately predict the increased TNB and T cell infiltration. In addition, TNB was significantly associated with the expression levels of M1 polarized macrophage genes, namely PD-1, PD-L1, interferon-γ (IFNγ), Granzyme B FAS ligand and other immune-associated genes (64). The correlation between TNB and TILs has been verified in multiple studies (64–66).

High TNB correlates with the abundance of TCR clonality and the infiltration of activated $CD4^+$ and $CD8^+$ T cells (50, 67). This may be mediated by the elevated expression of chemokines induced by IFN-γ, such as chemokine (C-X-C motif) ligand (CXCL) 9, and by the recruitment of T cells or myeloid dendritic cells (45, 68, 69). In the majority of the cases, high TNB can predict inflammatory microenvironment and optimal immune response. The infiltration of different immune cell subgroups is commonly noted in a special spatial compartment termed tertiary lymphoid structure (TLS). The mechanism by which TLS responds to the tumor microenvironment is actively studied. A previous report indicated that transforming growth factor β1 induced co-expression of CXCL13 and CD103 in $CD8^+$ T cells, providing a potential link between $CD8^+$ T cell activation and B cell migration (70, 71).

THE ROLE OF TNB IN TUMOR IMMUNOTHERAPY AND OTHER THERAPIES

Tumor Vaccine and T Cell Therapy

The use of individualized neoantigen vaccines and neoantigen-specific T cell therapy is actively explored. This topic has been well described in previous review articles (72–76) and will not be covered in the present review. It should be noted that individualized vaccines against a single neoantigen demonstrated limited efficacy. The use of a complete tumor lysate vaccine or a personalized vaccine containing multiple neoantigens can improve patient outcomes (6, 77). The dendritic cells were pulsed with oxidized autologous whole-tumor cell lysate, which was proved as an effective vaccine in patients with ovarian cancer. This vaccine amplified T cell responses against recognized neoepitopes and elicited *de novo* responses for previously unrecognized neoepitopes (78). An additional study tested the efficacy of an adenoviral vaccine consisting of multiple neoantigens. This vaccine facilitated T cell infiltration and expanded the breadth and efficacy of the TCR repertoire following ICI treatment (79). Tumor cell lysates differ in their efficiency as vaccines. A direct comparison of 2 autologous tumor cell lysate (with different TMB) vaccines demonstrated that the lysates with lower TMB inhibited tumor growth more efficiently. Thus, it may be considered that the neoantigen quality outranked the quantity (80).

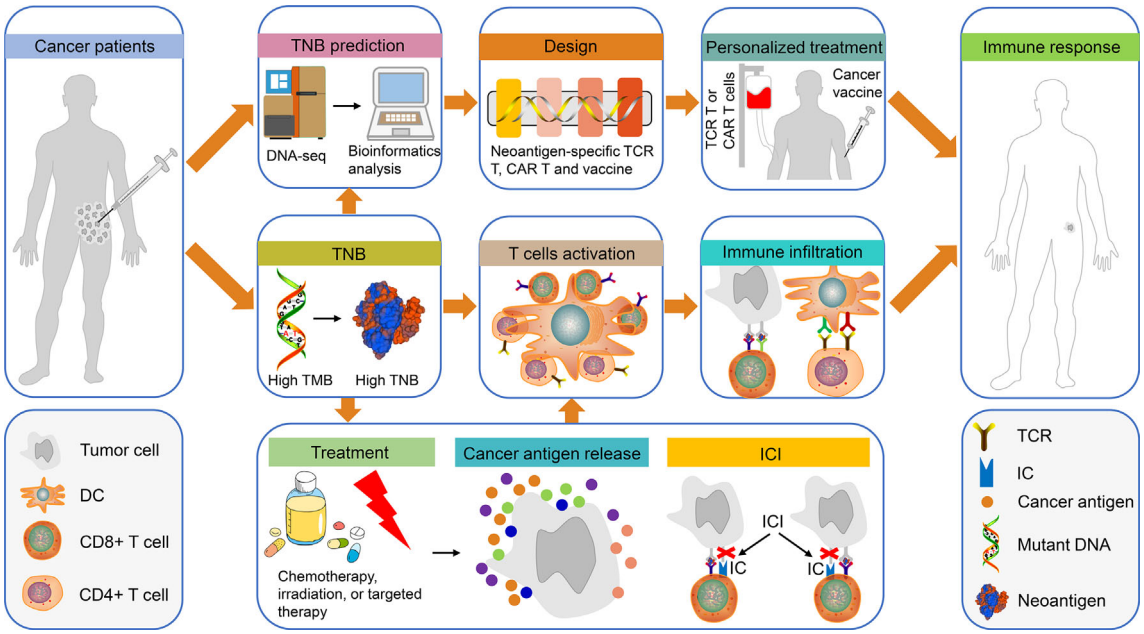

FIGURE 1 | The use of TNB in personalized immunotherapy for patients with cancer. Tumor tissues from patients with cancer were obtained for DNA sequencing and bioinformatics prediction of TNB. Subsequently, the TCR-T, CAR-T and the cancer vaccines were designed based on TNB for personalized treatment. Finally, the patients exhibited an immune response to cancer cells. In addition, high TMB in patients with cancer can produce high TNB, which will promote T cell activation and immune cell infiltration, thereby causing an immune response in these patients. It is interesting to note that chemotherapy, radiotherapy or targeted therapy can promote the release of cancer antigens in patients with high TNB, which in turn can enhance the therapeutic effects of ICI-based treatment. This type of treatment will activate T cells, increase immune infiltration and produce immune responses. TNB, tumor neoantigen burden; TCR-T, neoantigen-specific T cell receptor engineered-T cell; CAR-T, chimeric antigen receptor-T cell; ICI, immune checkpoint inhibitor.

Radiotherapy and Chemotherapy

Radiotherapy or chemotherapy can facilitate immunotherapy and is possibly attributed to increased exposure of neoantigens (81) (**Figure 1**). In addition to direct tumoricidal effects, radiotherapy converts the irradiated tumor cells into an *in situ* vaccine (82, 83). In locally advanced rectal cancer, neoadjuvant chemoradiotherapy induced new neoantigen epitopes and altered the immune function of the hosts (84). Similarly, patients with relapsed anal squamous cell carcinoma exhibited high TNB following radiochemotherapy and indicated objective responses to PD-1 inhibitors (85). In bladder cancer, dual poly (ADP-ribose) polymerase and PD/PD-L1 inhibition is used to improve disease prognosis (86). The standard treatment for high-grade serous ovarian carcinoma is surgery and/or chemotherapy. However, only dismal results are obtained, whereas in a subgroup of patients harboring high TNB, an improved prognosis was achieved. Moreover, an additional study demonstrated that TNB could be used to determine the prognosis of patients with clear cell renal cell carcinoma who received either surgery alone or surgery combined with adjuvant therapies (87–89).

The more important aspect is that pediatric tumors exhibit low TMB at diagnosis, whereas the levels of this biomarker increase when the tumor is exposed to chemoradiotherapy, resulting in neoantigen targets (90, 91). The majority of the pediatric tumors have less TIL and low MHC expression. In addition, the immune system of the children is immature. Consequently, the current immunotherapy alone is not sufficient to treat pediatric tumors efficiently. The combination of immunotherapy with conventional radio- and chemotherapy can achieve an improved survival benefit (92, 93).

TNB and Responses to ICIs

High TNB produces neoantigens, contributing to an inflammatory microenvironment, which ultimately leads to improved outcomes following ICI therapy (**Figure 1**). A previous study performed in patients with NSCLC, who exhibited high TMB or genetic defects in the DNA repair pathway, demonstrated that they benefited from ICI treatment. At least in one responder, neoantigen-specific $CD8^+$ T cell responses paralleled with tumor regression (26). It has also been shown that patients with melanoma, who are treated with CTLA4 inhibitors, demonstrated a significant association of TMB, TNB and cytolytic marker expression with clinical benefit (27). Recently, a model of immunotherapy score (ITS) mutation was proposed for predicting the response of patients with melanoma to ICI treatment. Patients with high TMB and TNB exhibited higher ITS scores and immunotherapy sensitive features (94).

A scoring system based on neoantigen concentration combined with clonality and MHC binding affinity predicted responses to ICIs and the prognosis of patients with melanoma,

lung and kidney cancers (39). Neoantigen concentration levels were prognostic factors for patients with melanoma and chronic lymphocytic leukemia treated with ICIs (29). However, paradoxical results were reported, indicating inferior PFS with high TNB in patients with multiple myeloma. The possible explanation for these findings was that the disease progression caused reduced efficiency of T cell recruitment (24).

The diversity and clonality of neoantigen-responsive TCR are also potential biomarkers for immunotherapy (95, 96). Additional research studies in this topic will aid the successful application of immunotherapy.

CHALLENGES AND PERSPECTIVES

The rapid development of immunology and bioinformatics has enabled the successful prediction of neoantigens. However, the standard pipeline for neoantigen prediction and the optimized cut-off value for TNB are unknown, since it is an emerging biomarker. In addition, the presence of specific mutations causing ITH should be taken into consideration (97). Heterogeneity exists not only locally but also between primary lesions and their successive metastases (98). Neoantigen prediction is currently hindered by the difficulty in exploring the entire tumor through a partial biopsy (99).

Several obstacles hinder the patient immune response, such as the loss of HLA (100–103). Failure of successful HLA presentation renders the candidate neoantigens ineffective *in vivo*. Therefore, TNB alone cannot accurately predict the immune response. Currently, the personalized detection of circulating tumor DNA is considered a powerful tool for the dynamic monitoring of TNB (16, 80, 104). However, the clinical application of TNB is still limited. Previous evidence has shown that the quality of neoantigens may be more important, since high-quality neoantigens can confer higher immunogenicity (105). According to our opinion, the real-time status of the high-quality neoantigen burden can monitor the treatment response more effectively. The construction of a neoantigen vaccine library and a neoantigen-responsive T cell receptor repertoire can provide a more comprehensive and personalized antitumor treatment. Future studies should focus on assessing the quality of TNB.

AUTHOR CONTRIBUTIONS

CW and PW conceptualized the study. PW wrote and edited the manuscript. YC helped revise the manuscript. All authors contributed to the article and approved the submitted version.

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Improvement of Neoantigen Identification Through Convolution Neural Network

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Accurate prediction of neoantigens and the subsequent elicited protective anti-tumor response are particularly important for the development of cancer vaccine and adoptive T-cell therapy. However, current algorithms for predicting neoantigens are limited by *in vitro* binding affinity data and algorithmic constraints, inevitably resulting in high false positives. In this study, we proposed a deep convolutional neural network named APPM (antigen presentation prediction model) to predict antigen presentation in the context of human leukocyte antigen (HLA) class I alleles. APPM is trained on large mass spectrometry (MS) HLA-peptides datasets and evaluated with an independent MS benchmark. Results show that APPM outperforms the methods recommended by the immune epitope database (IEDB) in terms of positive predictive value (PPV) (0.40 vs. 0.22), which will further increase after combining these two approaches (PPV = 0.51). We further applied our model to the prediction of neoantigens from consensus driver mutations and identified 16,000 putative neoantigens with hallmarks of 'drivers'.

Keywords: neoantigen, CNN, HLA, driver mutation, prediction

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INTRODUCTION

Cancer develops as a result of the accumulation of tumor-specific somatic mutations (1–3), where non-silent mutations in the coding region could be recognized as beacons of "foreign" by the immune system, named neoantigen (4, 5). They can elicit a protective anti-tumor response when presented on the surface of cancer cells by the major histocompatibility complex (MHC) [also called human leukocyte antigen (HLA)]. Neoantigens have long been regarded as ideal targets in immunotherapy because they are restrictedly expressed by tumor cells and not subjected to central or peripheral tolerance (6). Neoantigen-based immunotherapy has achieved great success in recent years (7–11), further highlighting the importance of accurate prediction of neoantigens for the development of cancer vaccines and adoptive T-cell therapy (12–15). However, the current prediction approaches and algorithms to identifying immunogenic neoantigens from mutant peptides are far from satisfactory. Low precision is a major obstruction to their identification scheme (16), partially because they primarily rely on the HLA-peptide binding affinity (17). The binding affinity produced by *in vitro*

binding experiments neglects other biological steps involved in the peptide delivery process, which results in a substantial fraction of false positives. Only ~1–5% of predicted bound peptides using HLA binding-affinity predictions have been experimentally validated (18). One way to solve this problem is to train the prediction algorithm with peptides eluted from HLA complexes of mono-allelic or mixed-allelic cancer cell lines and identified by mass spectrometry (MS) analysis (19). The MS datasets profile the peptides naturally presented on the cell surface, which has already gone through antigen processing and transporting steps (20, 21). Another reason for low precision may be that the recognition features, such as amino acid properties and spatial structure were not taken into consideration (22, 23). Compared with other artificial neural networks used in MHCflurry, NetMHC-4.0 and NetMHCpan-4.0 (24–26), the convolutional neural network (CNN) preserves local spatial features (27) and is more suitable for studying peptides where spatial locations of the amino acids are critical for binding (28).

In this study, we proposed an antigen presentation prediction model (APPM), a CNN algorithm trained to accurately predict the likelihood of a peptide presented by HLA-I molecules. APPM outperformed the approach recommended by IEDB (2020.04 netMHCpan EL 4.0) in terms of specificity and positive predictive value among 20 high-frequency HLA alleles. Besides, we predicted the neoantigens derived from the TCGA driver mutations, the preparation of which can be used in off-the-shelf immunotherapies to save the time from detecting mutations to personalized vaccine injection.

METHODS

Data Collection

More than 1,900,000 published HLA-peptides MS data of mono-allelic or mixed-allelic cell lines which collectively expressed 20 high-frequency HLA-A and HLA-B allotypes are collected (16, 19, 29, 30). All these data are labeled in binary notation. Label=1 denotes MS-identified peptides (hits), whereas label=0 denotes peptides from the reference proteome (SwissProt) that were not detected *via* mass spectrometry.

Data Encoding

The training datasets are peptides with the length from 8-mer to 11-mer, which are represented by a one-letter amino acid alphabet (a total of 20 distinct amino acids, namely 'ACDEFGHIKLMN PQRSTVWY'). Such length range captures ~95% of all HLA class I-restricted peptides. To implement machine learning, the peptide sequences are vectorized by a one-hot encoding scheme. Peptides with multiple lengths (8-mer to 11-mer) were represented as fixed-length vectors by using a padded character 'Z'. Each amino acid and the padded 'Z' are encoded as a one-hot vector (see **Figure S1** for details). As a result, peptides are encoded as the fixed matrix of 11 rows (maximum length) by 21 columns (20 distinct amino acid alphabets and the padded character 'Z').

Imbalanced Distribution of Training Datasets

The collection of MS datasets shows a severe class imbalance. Overall, the total number of 0-labeled data is 1,866,484 which is 39 times as many as the 1-labeled counterparts. An extreme case can be found in HLA-A*02:07 datasets where the negative-labeled records are 72 times more than 1-labeled records. Such extreme imbalance influences the prediction of the machine learning model, inclined to show a better performance on the 0-labeled peptides (the majority) and a worse on the 1-labeled ones (the minority) (31). Thus, the class balance is adjusted *via* over-sampling and under-sampling procedures in preprocessing the training datasets. Briefly speaking, the under-sampling goes by removing the 0-labeled training data points at random, whereas the over-sampling duplicates the 1-labeled data points. **Table 1** shows the proportions of over-sampling and under-sampling on different HLA alleles.

Convolutional Neural Network (CNN)

Usually, the Convolutional Neural Network (CNN) consists of convolutional layers, pooling layers and fully connected (dense) layers. In this study, an advanced CNN which is inspired by the *inception* module from *GoogLeNet* is used (32, 33). Three parallel convolutional sections with eight two-dimensional convolutional kernels for each were constructed to maximize the feature extraction (see **Figure S2** for details). The output of three convolutional layers connects to a flattened matrix and is delivered to the fully-connected layers which contain 100 hidden nodes. The output layer displays the results of binary classification by two nodes where a tested peptide is classified as binding or not binding to HLA.

The model is implemented with Tensorflow (v. 1.14.0) and trained by Adam optimization algorithm with standard parameters on an NVIDIA GeForce RTX 2080 Ti GPU. Instead of the frequently-used activation function Rectified Linear Unit (ReLU), the advance function of Leaky ReLU (α=0.2) is applied to activate the model and the "drop-out" and "early stopping" schemes are introduced to avoid overfitting.

Data Splitting

The peptides of the MS dataset are randomly split into training sets, validation sets and test sets, and all three sets have approximately the same distribution of 1-labeled and 0-labeled peptides. The validation sets are used only for early stopping. The training sets are used to perform feed-forward and backpropagation and the test sets are used to evaluate performance *via* AUC.

Independent Validation Dataset

To benchmark the APPM and other HLA-peptide predictors, we collected HLA-bound peptides MS datasets from other studies that use cell lines to express a single HLA allele (34, 35). From these MS-identified peptides (hits), we generated non-binders (decoy sets) by sampling unobserved peptides from the same proteins through the Uniprot human reference proteome (UP000005640_9606) as previously described (36). For each MS-identified peptide, we randomly selected 99-time decoy peptides of four different lengths (8, 9, 10, 11), and the number of each length is the same. The rationale for the 99-fold bias is that for a sample of peptide fragments from an organism, it is commonly considered that approximately 1%~2% of the fragments will bind to MHC receptors (37). After removing the peptides appearing in the model training data and the

TABLE 1 | The Training Detail on different HLA alleles.

Alleles	Label = 1	Label = 0	Train	Test	Under-sampling	Over-sampling
A*01:01	3398	48700	45498	6600	1	2
A*02:01	6779	165342	160921	11200	0.8	3
A*02:03	1780	116299	107879	10200	0.8	3
A*02:07	3206	232783	225389	10600	0.7	5
A*03:01	5419	83117	77536	11000	1	3
A*11:01	2114	123143	114857	10400	0.8	3
A*24:02	5189	142382	136571	11000	0.7	3
A*29:02	1149	54125	49074	6200	1	5
A*31:01	1879	45918	41597	6200	1	4
A*32:01	584	40401	34885	6100	1	5
A*68:02	1516	92678	83994	10200	0.8	3
B*07:02	3162	201778	194340	10600	0.6	3
B*15:01	1684	106482	97966	10200	0.8	3
B*35:01	1019	53819	48638	6200	1	4
B*40:01	1321	80192	71313	10200	0.9	3
B*44:02	1525	44760	40085	6200	1	4
B*44:03	1487	39482	34769	6200	1	4
B*51:01	2597	77898	70095	10400	1	4
B*54:01	969	65623	56412	10180	1	3
B*57:01	1599	51562	46961	6200	1	4

Alleles defined by DNA sequencing are named to identify the gene, followed by an asterisk, numbers representing the allele group.

duplicate sampled from different proteins, we obtained a mono-allelic benchmark dataset.

Predictive Performance Metric Calculation

Sensitivity, also called recall, was calculated as:

$$\frac{correctly\ \ predicted\ \ positive\ \ peptides}{all\ \ positive\ \ peptides}$$

Specificity was calculated as:

$$\frac{correctly\ \ predicted\ \ negative\ \ peptides}{all\ \ negative\ \ peptides}$$

Positive predictive value, also called precision, was calculated as:

$$\frac{correctly\ \ predicted\ \ positive\ \ peptides}{all\ \ peptides\ \ predicted\ \ to\ \ be\ \ positive}$$

The Cancer Genome Atlas (TCGA) Driver Mutations

To obtain a consensus driver mutations list, we download the driver-mutations dataset processed and compiled by TCGA MC3 and driver working group (https://gdc.cancer.gov/about-data/publications/pancan-driver) (38, 39). The driver-discovery dataset was derived from a compiled MAF file of 9079 TCGA samples across 33 different cancer types (syn7824274, https://gdc.cancer.gov/about-data/publications/mc3-2017). Based on sequencing and structure analyses, we ultimately selected 3,437 cancer driver mutations as the consensus list were identified by ≥ 2 approaches from CTAT-population, CTAT-cancer, or structural clustering (see **Supplementary File 4**).

Candidate Peptides From Driver Mutations

For each driver mutation, we extract 8-11mers candidate peptides that contain the driver specific mutant amino acid for neoantigen screening. For instance, the extracting procedure of 9-mer candidate peptides is described as follows (**Figure S3**). Firstly, we extracted a 17-mer peptide from the protein sequences, where the mutant amino acid was placed in the center with eight upstream and downstream wild amino acids as flanks. Secondly, by using the sliding window protocol, a 9 amino acid size window was slid N (N = 9) times to obtain 9-mer peptides. Briefly speaking, the mutant amino acid serves as the end point of the first 9-mer peptide. This 9-mer sliding window moves along the 17-mer fragment until the mutated point becomes the starting point of the 9-mer. Peptides with other lengths are treated in the same way.

RESULTS

Development of APPM

We aimed to improve the precision and specificity of the HLA-peptide prediction approaches through a novel tool that has been trained on improved training data and a new supervised machine learning model. HLA-Peptides of MS data were eluted by immunoprecipitation of HLA molecules and then identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (40, 41). Compared with *in vitro* binding affinity assays, MS data directly profiles peptides that are actively presented by cells or tissues (42). We collected publicly available HLA-peptides MS data from 16 mono-allelic HLA-A and HLA-B cell lines genetically engineered to express a single HLA allele and from B lymphocytes or cancer cell lines expressing multiple HLA complex alleles (16, 19, 29, 30, 43). These MS data consist of 20 high-frequency HLA-I alleles. We split the datasets into three sets: training, validation and testing sets (Methods). Owing to so many negative peptides (from reference proteome), we apply the over-sampling and under-sampling scheme, which neutralizes the substantial fraction of the imbalance issue.

Using these public HLA-peptides MS data, we build a convolutional neural network (CNN) framework to predict HLA-I presentation, a form of deep learning that excels at handling general sequence data such as amino acid sequences (**Figure 1**) (28). The model has three parallel convolutional modules, each consisting of eight two-dimensional convolutional layers, which preserved HLA class I-peptide binding features.

Predictive performance of APPM

To estimate the predictive performance of APPM, we first compared the prediction results of APPM with the IEDB recommended method (2020.04) (NetMHCpan4 EL (44), the state-of-the-art class I binding predictors available at http://tools.iedb.org/mhci/) in terms of PPV. We compiled a benchmark using published MS data from cell lines genetically engineered to express a single HLA-I allele. In this mono-allelic benchmark, the MS-identified peptides are true positives where length-matched amino acid fragments from the same protein as negative peptides (decoys). For each paired HLA allele and peptide, NetMHCpan4 EL produced a binding score and percentile ranks. Using the recommended threshold of the percentile rank (top 2% ranks are considered binders), we obtained the average specificity and positive predictive value (PPV) of 0.97 and 0.22 for NetMHCpan4 EL (**Supplementary File 1**).

When tested on the same data, APPM outperformed NetMHCpan4 EL with the specificity of 0.99 and PPV of 0.40. The improvement in reducing false positives rates was substantial, with an average of 80% increase in PPV (**Figure 2A**). For the 20 frequent haplotypes of HLA class I, APPM only exhibited a slightly lower PPV than NetMHCpan4 EL on HLA-A*02:01, but presented higher PPV for the rest of 19 HLA haplotypes, particularly with more than one fold of increase for HLA-A*02:03, HLA-A*29:02, HLA-A*32:01 and HLA-B*40:01 (**Figure 2B**), suggesting the advantage of our algorithm.

Combining Algorithms Improves Prediction Performance

Interestingly, a low overlap rate (19%) is observed between APPM and NetMHCpan4 EL for the false-positive peptides (**Figure 3A**), probably due to the different prediction mechanisms. In this case, we hypothesized that the prediction performance could be improved by combining these two predictive approaches. We redefined the predictive results: only peptides identified positively in both methods are regarded as positives. Using the combined predictions, we obtained the PPV of 0.51 (**Figure 3B**), which is significantly higher than that of both APPM and NetMHCpan4 EL (**Figure 3C**, $p = 0.013$, t-test and **Figure 3D**, $p < 0.001$, t-test), without significant decrease of sensitivity (**Figure 3E**, $p = 0.1$, ANOVA). These results suggested that the combined predictions from different algorithms can improve the positive rate for neoantigen selection, which is consistent with previous studies (45, 46).

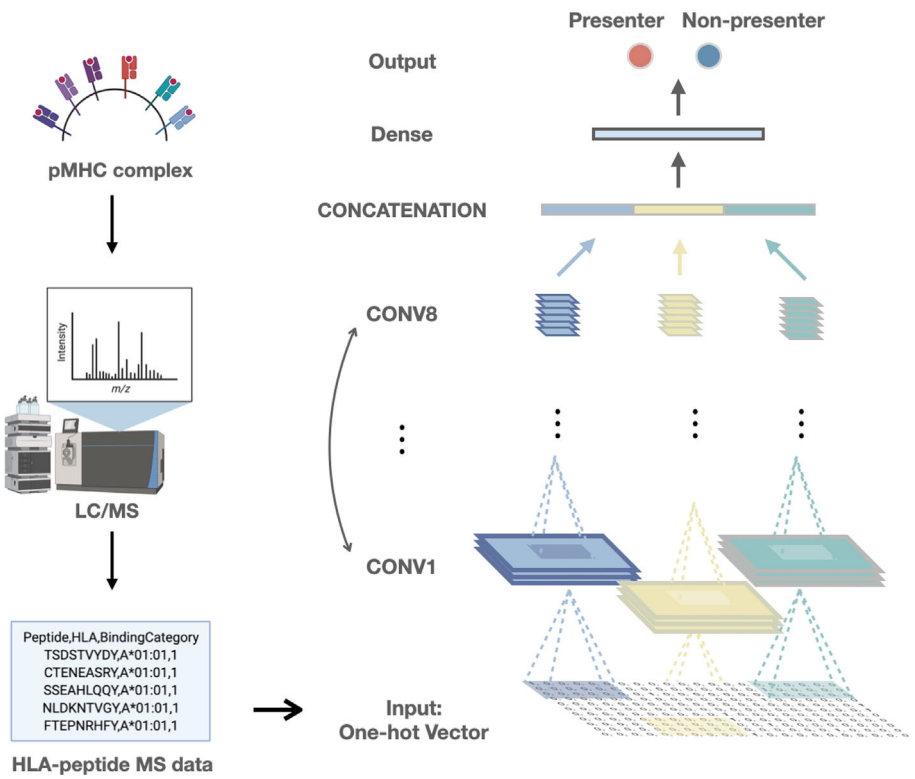

FIGURE 1 | The framework of our study includes the collection of training data and the deep learning model built based on the convolutional neural network.

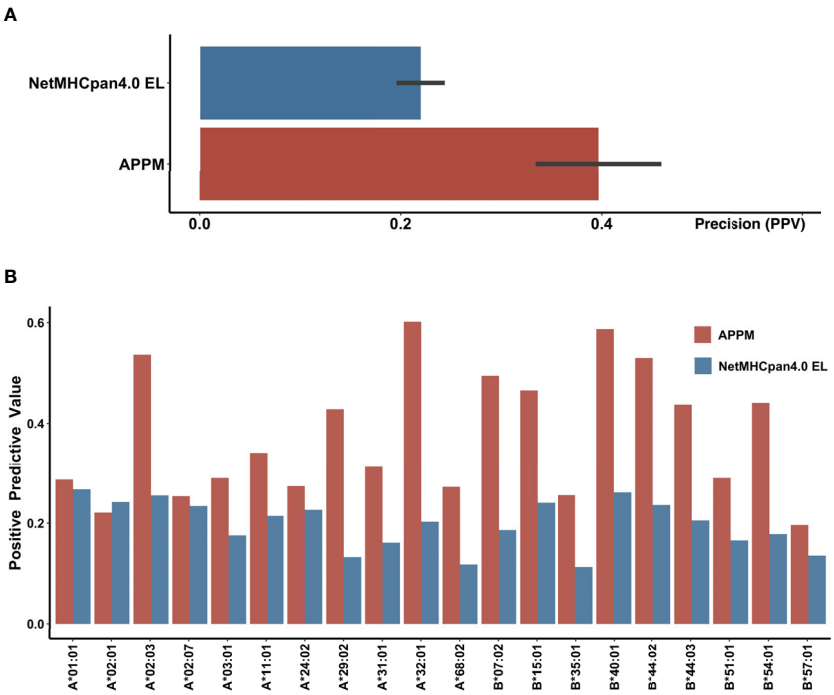

FIGURE 2 | Validation performance of IEDB recommended approach and APPM **(A)** The mean PPV accuracy on the mono-allelic MS benchmarks for APPM and NetMHCpan4 EL. **(B)** The PPV values of two predictors at different HLA alleles.

Alleles-Specific Presentation Motif

To illustrate the binding characteristics of HLA-I alleles with peptides, we draw allele-specific presentation motifs for 20 HLA-I alleles (see **Supplementary File 2** for motifs of all alleles). Consistent with previous studies (17, 19, 47), these motifs revealed the dependence of HLA presentation on each sequence position for peptides of multiple lengths 8-11 (**Figure 4A**). For example, the anchor residues of 9mer are amino acid at position 2 (refer as P2, a similar abbreviation for other positions) and P9, while 11mer at P2 and P11.

In contrast to previous work (48), some distinct HLA alleles have similar presentation motifs. For instance, HLA-A*02:01 and HLA-A*02:03 have the same binding specificity, meaning the pockets preferentially bind to bind the peptides with leucine at P2 and valine/leucine at the last position. Likewise, HLA-A*03:01 and HLA-A*11:01 presented lysine at the last position, while HLA-B*40:01, HLA-B*44:02, and HLA-B*44:03 prefer to deliver peptides with glutamate at P2 (**Figure 4B**).

Moreover, we analyzed the amino acid properties of anchor residues of 20 HLA alleles and refined their binding character: these binding peptides enriched in hydrophobic amino acids at anchor residues. It is consistent with the known preference of HLA-I binding and presentation (23, 49). We also explored the whole preference of amino acid properties among HLA-A and HLA-B molecules on anchor residues (**Figure 4C**). Besides the common preference of hydrophobic amino acids, HLA-A alleles prefer to bind basic and polar amino acids, while the HLA-B alleles prefer acidic amino acids.

Neoantigens From Driver Mutations

It is considered that the quality rather than the quantity of neoantigens may lead to a robust and durable response to immunotherapy (50). Most of the putative neoantigens are considered as the product of passenger rather than driver mutations, and their loss through chromosomal instability during tumor evolution may be readily tolerated. Therefore, targeting driver-mutation-neoantigens could manifest durable anti-tumor responses and may reduce the resistance to neoantigen therapies.

We applied the combining approach of APPM and NetMHCpan4 to predict neoantigens derived from oncogenic driver mutations. The consensus driver-mutation list was compiled and discovered by The Cancer Genome Atlas (TCGA) Multi-Center Mutation Calling in Multiple Cancers (MC3) working group and driver working group among 9079 samples across 33 cancer types (38, 39). For a total of 3,437 missense driver mutations, we identified ~ 16,000 putative neoantigens in the context of 20 high-frequency HLA alleles (**Supplementary File 3**).

Among these driver mutations, only 15% (513/3437) do not yield putative neoantigens, while the products of the other could be bound and presented by these HLA alleles. We identified 36 high-frequent shared putative neoantigens derived from eight

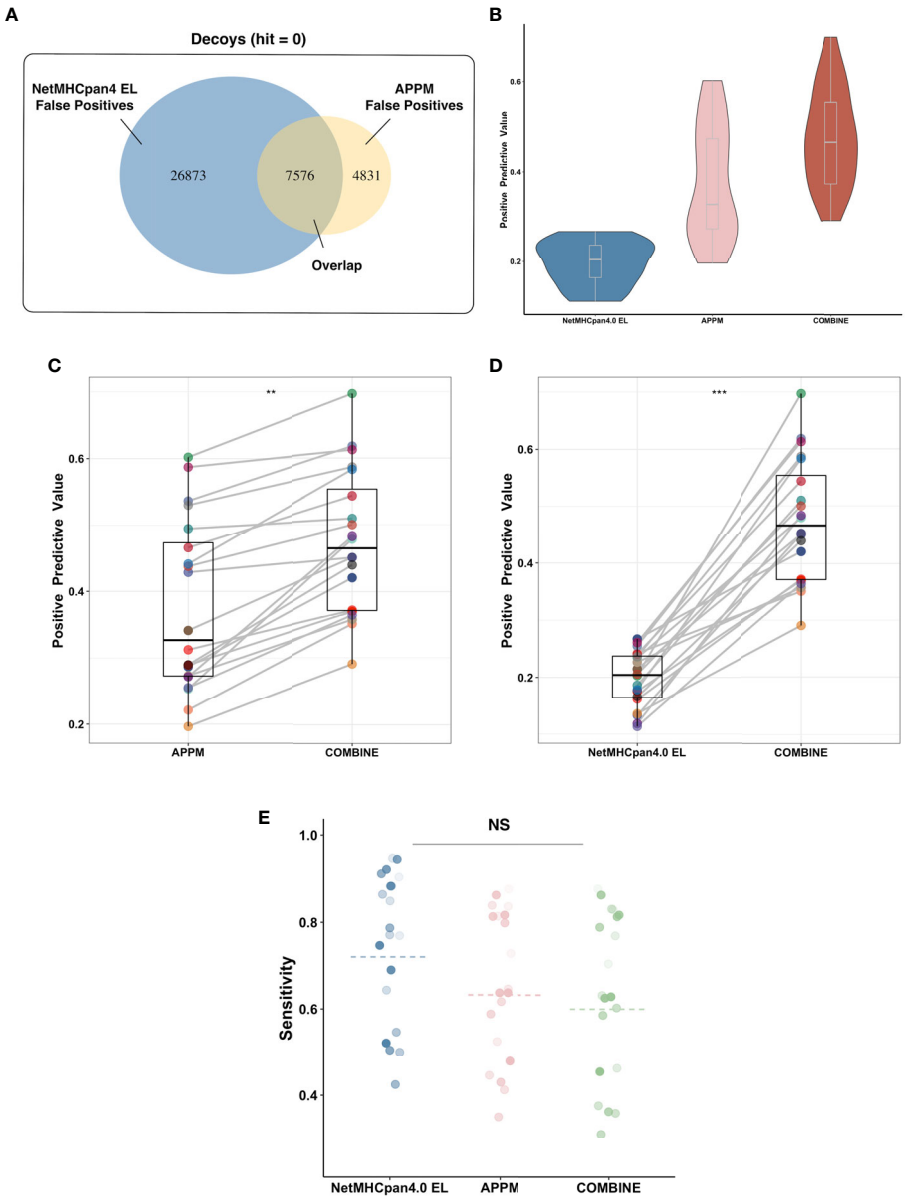

FIGURE 3 | Algorithms Combination Improves Prediction Performance. **(A)** The false-positive peptides of APPM and NetMHCpan4 EL. These peptides are decoy peptides of mono-allelic MS benchmarks that are incorrectly predicted to be bindings. **(B)** The mean PPV accuracy on the mono-allelic MS benchmarks for APPM, NetMHCpan4 EL and combination. **(C)** The significant improvement of predictive performance in the term of PPV on the mono-allelic MS benchmarks. The left is APPM and the right is the combination of APPM and NetMHCpan4 EL. **p < 0.05. **(D)** The significant improvement of predictive performance in the term of PPV on the mono-allelic MS benchmarks. The left is NetMHCpan4 EL and the right is the combination of APPM and NetMHCpan4 EL. ***p < 0.01. **(E)** The mean sensitivity on the mono-allelic MS benchmarks for APPM, NetMHCpan4 EL and combination. NS, no significance.

oncogenic driver mutations with more than 1% coverage of multiple cancer patients in the 9079 TCGA cohort (**Table S1**), e.g. HLA-A*03:01_KIGDFGLATEK from BRAF_p.V600E with 5.60% (508/9079) in Pan-Cancer. Besides, we also found tumor-specific shared potential neoantigens with over 10% frequency in a given cancer type (**Table S2**). For example, HLA-B*15:01_IIIGCHAY from IDH1_p.R132C with 11.76% (4/34) in CHOL. Importantly, the immunogenicity of some shared putative neoantigens we identified has been confirmed experimentally (**Table 2**) (51). For instance, VVVGAGDVGK from KRAS_p.G13D has been shown to be immunogenic in the context of the HLA-A*03:01 allele. Overall, these putative shared driver-mutation-neoantigen pools provide a potential list of targets for off-the-shelf immunotherapy.

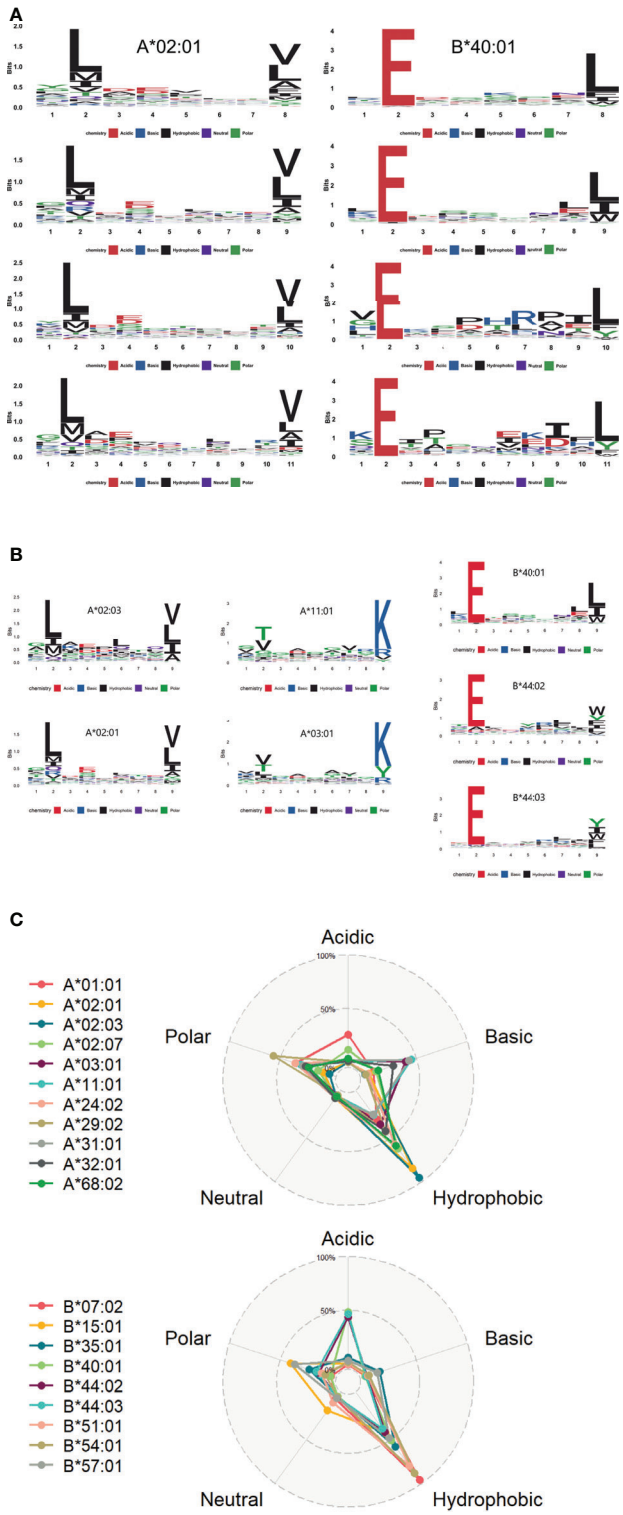

FIGURE 4 | The motif of HLA alleles **(A)** The learned dependence of HLA presentation on each sequence position for peptides of lengths 8–11. The red, blue, black, purple, and green lines represent the acidic, basic, hydrophobic, neutral and polar amino acids respectively. **(B)** Some similar motifs are depicted in this graph. **(C)** The radar view is a deformation of the percentage graph illustrating the motifs of HLA-A and HLA-B at the overall level. Different colors represent varied HLA class I molecules. Alleles defined by DNA sequencing are named to identify the gene, followed by an asterisk, numbers representing the allele group.

TABLE 2 | Validated immunogenic neoantigens derived from driver mutations.

Driver Mutation	pmhc	CancerTypes	Frequency
KRAS_p.G12D	HLA-A*03:01_VVGADGVGK	Pan-Cancer	1.78% (162/9079)
KRAS_p.G13D	HLA-A*03:01_VVVGAGDVGK	COAD	8.77% (20/228)
KRAS_p.G13D	HLA-A*03:01_VVGAGDVGK	COAD	8.77% (20/228)
KRAS_p.Q61H	HLA-A*01:01_ILDTAGHEEY	PAAD	3.87% (6/155)
KRAS_p.Q61L	HLA-A*01:01_ILDTAGLEEY	TGCT	1.55% (2/129)
KRAS_p.Q61R	HLA-A*01:01_ILDTAGREEY	COAD	1.32% (3/228)
IDH2_p.R140Q	HLA-B*07:02_SPNGTIQNIL	LAML	4.35% (6/138)

DISCUSSION

Neoantigen is the foreign protein that arises as a consequence of tumor-specific DNA alterations and could be presented on the surface of tumor cells by MHC molecules. When recognized by TCR specifically, it will elicit anti-tumor immune responses. In the current clinical application of targeting neoantigens immunotherapies, the accurate identification of relevant neoantigens has become a central challenge (46). Current prediction algorithms are insufficiently precise due to the limitation of *in vitro* binding affinity training data and algorithmic constraints, therefore resulting in high false positives (16, 19, 41). One of the solutions is to train a novel prediction algorithm by using MS-identified peptides from mono-allelic or mixed-allelic cell lines (19, 52).

In this study, we build high PPV neoantigen prediction algorithms by training models on *in vitro* MS data and CNN deep learning model. Based on the mono-allelic benchmark, we demonstrate that our model, APPM, outperforms netMHCpan4 EL among 19 high-frequency HLA alleles in precision. Moreover, the combination of APPM and NetMHCpan4 EL improves the prediction performance, suggesting that the combined strategy can identify potential neoantigens in clinical practices with more precision. However, the mass spectrometry assay itself has a technological limitation: not all possible eluted ligands can be detected, which inevitably generates the false negative peptides (53–55).

An important limitation of this work is that we apply MS datasets to train and evaluate our predictor. Using MS-identified peptides to reflect the factor of gene expression, protease cleavage, transportation and presentation might bring the MS bias in our prediction. Our work also neglects T cell recognition of presented epitopes. Many putative neoantigens identified by our predictor will not induce CD8+ T cell responses when used in cancer patients. This limitation is consistent with the previous study that presentation of antigens is essential but not sufficient for induction of robust anti-tumor responses (56).

Besides, neoantigens derived from driver mutations are particularly important for neoantigen-targeting immunotherapy. Firstly, driver-mutation-neoantigens are a source of "high-quality neoantigens" that may reduce the likelihood of resistance to neoantigen therapy. Secondly, driver mutations were shared between patients of the same cancer type with relatively high frequencies (57–61), as well as between primary tumors and metastases (62). A limited number of high-frequent driver mutations may generate shared neoantigens that could be widely applied to multiple tumor patients and may be ideal targets for off-the-shelf immunotherapy (63). However, whether the shared putative neoantigens are immunogenic in different cancer patients remains to be determined. Nevertheless, prioritizing such neoantigens whenever possible is important, as constructing a library for storage of these shared neoantigens can significantly save time from detecting mutations to the preparation of the personalized vaccine and increase the efficiency of neoantigen-based immunotherapies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors. All training data and code are available on Github at: https://github.com/haoqing12/APPM.git.

AUTHOR CONTRIBUTIONS

QH trained the model and wrote the manuscript. PW, YS, Y-GZ, HX, and J-NZ reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.682103/full#supplementary-material

Supplementary Figure 1 | Example of the peptide sequence 'ARHSLLQTL' using one-hot encoding scheme.

Supplementary Figure 2 | The full CNN model structure. Purple, yellow and green represent three parallel convolutional layers. The black box represents the convolution kernel of each layer.

Supplementary Figure 3 | The extracting procedure of candidate peptides. The blue points represent the wild amino acids and the red points refer to the driver mutant amino acids.

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REVIEW
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Mechanisms of Neoantigen-Targeted Induction of Pyroptosis and Ferroptosis: From Basic Research to Clinical Applications

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Neoantigens are tumor-specific antigens (TSAs) that are only expressed in tumor cells. They are ideal targets enabling T cells to recognize tumor cells and stimulate a potent antitumor immune response. Pyroptosis and ferroptosis are newly discovered types of programmed cell death (PCD) that are different from apoptosis, cell necrosis, and autophagy. Studies of ferroptosis and pyroptosis of cancer cells are increasing, and strategies to modify the tumor microenvironment (TME) through ferroptosis to inhibit the occurrence and development of cancer, improve prognosis, and increase the survival rate are popular research topics. In addition, adoptive T cell therapy (ACT), including chimeric antigen receptor T cell (CAR-T) technology and T cell receptor engineered T cell (TCR-T) technology, and checkpoint blocking tumor immunotherapies (such as anti-PD- 1 and anti-PD-L1 agents), tumor vaccines and other therapeutic technologies that rely on tumor neoantigens are rapidly being developed. In this article, the relationship between neoantigens and pyroptosis and ferroptosis as well as the clinical role of neoantigens is reviewed.

Keywords: neoantigens, pyroptosis, ferroptosis, chimeric antigen receptor T cell (CAR-T), T cell receptor engineered T cell (TCR-T), PD-1/PD-L1, tumor vaccine

INTRODUCTION

Tumors are a major threat to human life and health. Tumor antigens can be divided into tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs). The majority of tumor antigens are TAA-specific embryonic antigens. Different antigens have different levels of immunogenicity, not only low immunogenicity, and it is difficult to induce a long-lasting specific immune response. In addition, TAA-targeted therapy may break immune tolerance, resulting in activation of immune cells and attack of cells expressing self-TAAs in normal tissues, causing autoimmune responses and autoimmune disease. TSAs are generated by nonsynonymous point mutations, deletion and/or insertions, gene fusions, and frameshift mutations that generate neoantigens. Neoantigens are only expressed in tumor cells, not normal tissue cells. They are ideal targets enabling T cells to recognize cancer cells. They can stimulate a strong antitumor immune response and are a major factor in clinical immunotherapy. Neoantigens can serve as biomarkers in cancer immunotherapy (1). The

prerequisite for all treatments based on tumor neoantigens is the screening and identification of neoantigens (2). Therefore, identifying new antigens has become a top priority. The screening methods used to identify tumor neoantigens currently mainly include target gene sequencing (3), exome sequencing (4, 5), antigen ligandomics and mass spectrometry combination technology (4, 6, 7), and tandem micro gene sequencing methods (8, 9)(as shown in **Figure 1**).

Pyroptosis is a kind of programmed cell death (PCD) characterized by proinflammatory and lytic properties, and it is also an important immune defense response of cells. Pyroptosis is mainly divided into the classical pathway, which depends on caspase-1 (cysteine aspartase-1) to mediate pyroptosis, the nonclassical pathway, which depends on caspase-4/5/11 to induce pyroptosis, and a pathway by which activation of tumor drugs induces caspase-3-mediated cleavage of GSDME into GSDME-N, which leads to pyroptosis. There are three pathways (10). It is now generally accepted that GSDMD is the executor of pyroptosis. GSDMD-N forms tiny pores of 1.0-2.4 μm in the cell membrane, which makes the ion gradient inside and outside the cell unbalanced. Water continuously pours into the cell until the cell ruptures, and inflammatory effectors flow out (IL-18/IL-1β), further enhancing the inflammatory response and ultimately leading to cell pyroptosis (11–13). The morphology of cells undergoing pyroptosis includes a cytoplasm that is preferentially located towards the surface, a centered nucleus, and a flattened shape that looks similar to cabbage or a fried egg (14). The specific mechanisms of pyroptosis are as follows (15, 16) (**Figure 2**): (I) Bacteria, viruses, and inflammatory factors can activate NOD-like receptors (NLRs), absentin melanoma2 (AIM2) or pyrin domains (PYD) through pathogen-associated molecular patterns (PAMPs) or noninfectious stimulation-associated damage-associated patterns (DAMPs). Such activity caspase-1, and then cleavage GSDMD to perform the occurrence of pyroptosis for GSDMD-N, and meanwhile activate nod-like receptor protein 3 (NLRP3). Apoptosis associated speck-like protein containing aCARD (ASC) further activates caspase-1. (II) Lipopolysaccharide (LPS) could directly activate caspase-4/5/11, mediate the activation of GSDMD, activate NLRP3 inflammasome, and maturation and release of IL-1β and IL-18. (III) Chemotherapy drugs induce caspase-3 to cleave gasdermin E into GSDME-N and punch holes in the cell membrane to turn apoptosis into pyroptosis. It is worth mentioning that caspase-1 promotes the maturation of inflammatory cytokines IL-1β and IL-18, and the GSDMD-N terminal releases inflammatory substances and induces pyroptosis. So these two proteins are crucial in pyroptosis. It has been shown that the expression of pro-death could be inhibited by the ROS scavenging agent N-acetylcysteine (17). And melatonin, which has a strong anti-inflammatory effect, could also prevent endothelial cell pyroptosis by down-regulating gene expression related to pyroptosis through the lncRNA-MEG3/miR-223/NLRP3 signaling pathway (18). Dolma (19) and others identified ferroptosis as a new form of PCD, and ferroptosis has become a research hot spot. Ferroptosis, mainly caused by the balance between the generation and degradation of reactive oxygen species in lipids, is an iron-dependent form of cell death caused by metabolic dysfunction of lipid oxides in cells under the action of divalent iron or ester oxygenase. High expression of unsaturated fatty acids on cell membranes and accumulation of lipid reactive oxygen (ROS) species result in an imbalance of intracellular redox. Studies have shown that inflammation is closely related to ferroptosis and has been shown to play an important role in the pathogenesis of aseptic inflammation (e.g., ischemia-reperfusion injury, stroke, and non-alcoholic hepatitis) as well as microbial infectious diseases (20–23). It is mostly in the following ways (24–26): 1. Glutamine (Gln) enters the cytoplasm through SLC38A1 and SLC1A5 of the cystine/glutamate antiporter system (system Xc-) and is then converted into glutamate (Glu). Free Glu also enters the cytoplasm through SLC7A11 and SLC3A2. Under the action of selenocysteine, Glu and cysteine work together to produce reduced glutathione (GSH). Then, oxidized glutathione (GSSG) is generated through the action of glutathione peroxidase 4 (GPX4). More importantly, under the action of glutathione peroxidase 4 (GPX4), L-OOH is deoxygenated to L-OH. This process leads to the accumulation of lipid ROS, which leads to ferroptosis. 2. The conversion of Fe^{2+} to Fe^{3+} and the direct conversion of free Fe^{3+} to Fe^{2+} can be accomplished under the action of ferroportin (FPN). Fe^{3+} can also enter cells through transferrin receptor 1 (TFR-1). Then, in the prostate, under the action of six-transmembrane epithelial antigen of prostate 3 (STEAP3), Fe^{3+} is reduced to Fe^{2+}. Fe^{2+} is transported in cells by zinc transporter 8/14 (ZIP8/14) and divalent metal transporter 1 (DMT1). The resulting Fe^{2+} undergoes the Fenton reaction to induce the accumulation of lipid ROS, which leads to ferroptosis (**Figure 3**). Ferroptosis has unique characteristics in terms of cell morphology, genetics, and biochemistry. In terms of morphology, mitochondria become smaller in size, the membrane density increases, and cristae appear blurred or even reduced, and morphological changes in the nucleus are

Abbreviations: ACT, adoptive T cell therapy; AIM2, absent in melanoma; ART, artemisinin; ASC, apoptosis-associated speck-like protein containing Acard; CARD, Caspase activation and recruitment domain; Caspase, caspase family of proteins; CRT-T/TCR-T, chimeric antigen receptor T cell technology/T cell receptor engineering T cell technology; DAMPs, damage-associated molecular patterns (noninfectious stimuli); DMT1, divalent metal ion transporter; FPN, Membranotransferrin; GD2, disialoganglioside; GPX4, glutathione peroxidase 4; GSDME or E, Gasdermin E or D; GSH, glutathione; GSSG, oxidized glutathione; GVHD, graft and host disease; H-FIRE, High-frequency irreversible electroporation; IFN-γ, interferon-γ; IHC, immunohistochemistry; IIS, PD-1 or PDL-1 blockers; IL, interleukin; L-OH, the hydroxyl group of L-amino acids; LOOH, the carboxyl group of L-amino acids; LPS, lipopolysaccharide (gram-negative bacteria cell wall component); ASC, apoptosis-associated speck-like protein containing a CARD; MHC, histocompatibility complex; NLRP3, NOD-like receptor protein 3; NK-CAR, CAR-engineered NK; NK-TCR, TCR-engineered NK; PAMPs, pathogen-associated molecular patterns; PD-1/PD-L1, programmed death receptor/programmed death protein receptor ligand; pLNMS, pathological lymph node metastases; pMHC, peptide-major histocompatibility complex; PRR, pattern recognition receptor; PSMA, prostate-specific membrane antigen; PYD, pyrin domain; RIG-1, Retinoic acid-inducible gene I; ROS, reactive oxygen species; STING, stimulator of interferon genes; SystemXc-, cystine/glutamate reverse transporter system; TEM, tumor microenvironment; XCT, Cystine/glutamate antiporter; ZIP, Zinc transporter.

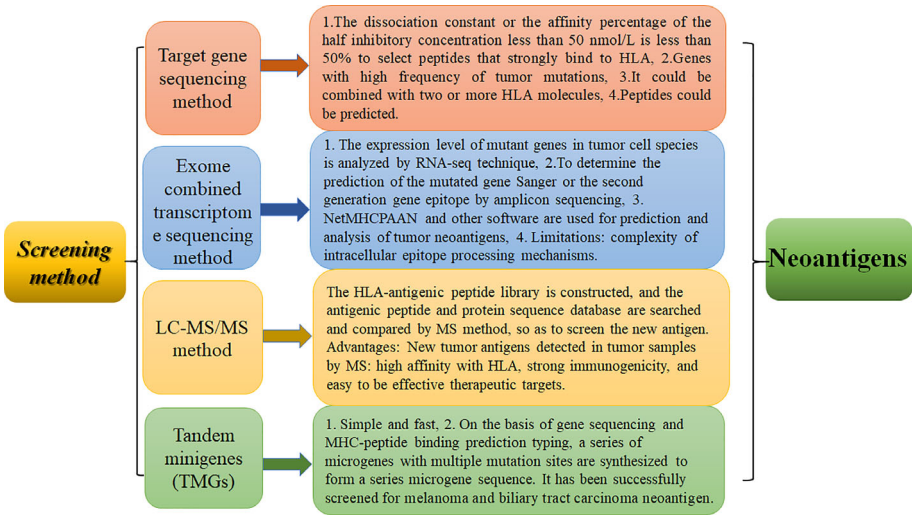

FIGURE 1 | Screening methods of neoantigen identification and their advantages and disadvantages. The advantages and disadvantages of four methods, including serological analysis of recombinant expression cDNA clones, exome combined transcriptome sequencing method, LC-MS/MS method, and tandem microgenre method, are discussed.

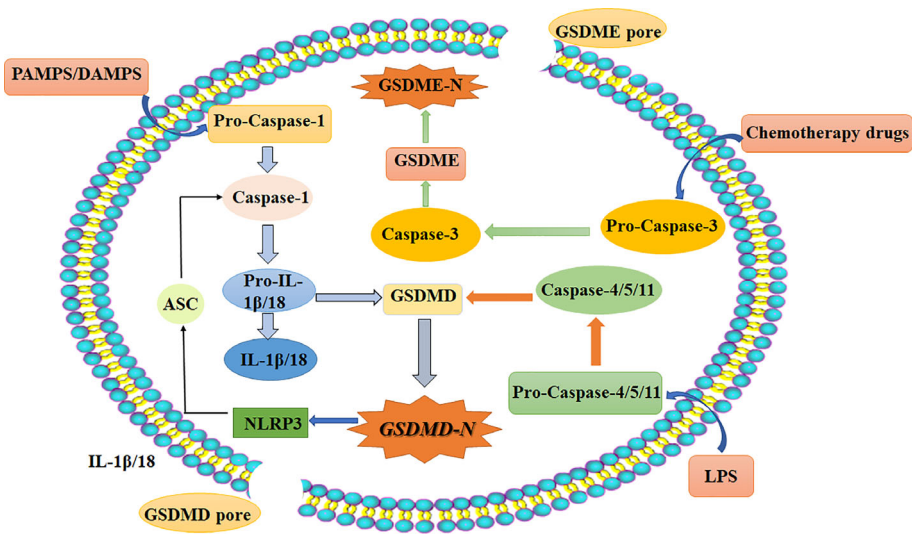

FIGURE 2 | Schematic diagram of the three main pyroptosis pathways: Classical pathways that rely on caspase-1/NLRP3/GSDMD; Independent of caspase-1, through the nonclassical pathway caspase-4/5/11; (1). Bacteria, viruses, and inflammatory factors can activate NOD-like receptors (NLRs), absentin melanoma2 (AIM2) or pyrin domains (PYD) through pathogen-associated molecular patterns (PAMPs) or noninfectious stimulation-associated damage-associated patterns (DAMPs). (2). Lipopolysaccharide (LPS) could directly activate caspase-4/5/11, mediate the activation of GSDMD, activate NLRP3 inflammasome, and maturation and release of IL-1β and IL-18. (3). Chemotherapy drugs induce caspase-3 to cleave gasdermin E into GSDME-N and punch holes in the cell membrane to turn apoptosis into pyroptosis. Chemotherapy-induced caspase-3/GSDME specific pathways. DAMPs, damage-associated molecular patterns (noninfectious stimuli); PAMPs, pathogen-associated molecular patterns; LPS, lipopolysaccharide (gram-negative bacteria cell wall component); ASC, apoptosis-associated speck-like protein containing a CARD; IL, interleukin; NLRP3, NOD-like receptor protein 3; caspase, caspase family of proteins; GSDMD, gasdermin D.

not obvious as major characteristics (27). In addition, in terms of biochemistry, the content of iron increases, ROS are produced in excess, and the expression levels of GSH and GPX4 are decreased. Furthermore, there are changes in some characteristics of genes (28). Studies show that iron chelating agents and antioxidants can inhibit the occurrence of ferroptosis, which can be induced by systemXc- inhibitors, GPXs inhibitors, and other compounds. The inductive mechanisms of ferroptosis

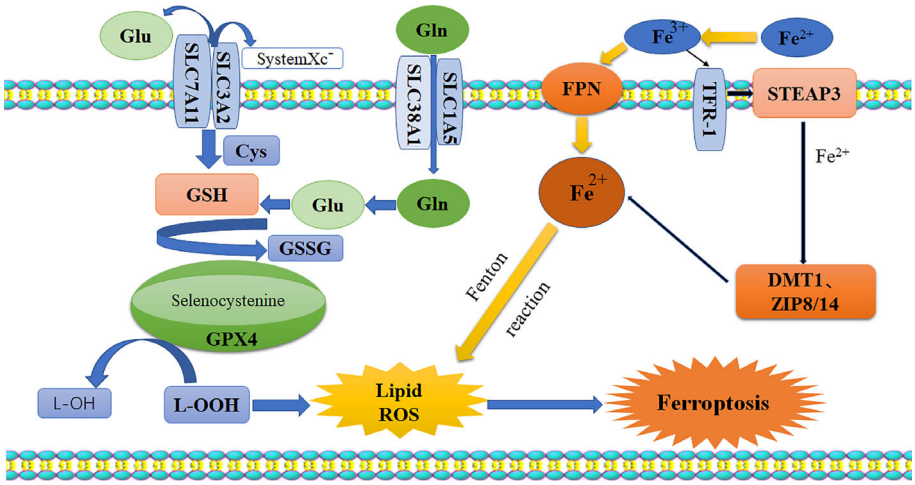

FIGURE 3 | Schematic diagram of ferroptosis pathway. Glutamate and glutamine enter the cell body through system-xc, while Fe^{2+} enters the cytoplasm through FPN. Their accumulation would break the balance in the cells, and the lipid ROS is generated by GPx4 and Fenton reaction, resulting in ferroptosis. Glu, glutamate; Gln, glutamine; Cys, cysteine; GSH, reduced glutathione; GSSH, oxidized glutathione; GPX4, glutathione peroxidase 4; ROS, reactive oxygen species; FPN, ferroportin; TFR-1, transferrin receptor-1; STEAP3, six-transmembrane epithelial antigen of prostate 3; DMTI, divalent metal transporter; ZIP8/14, zinc transporter 8/14.

in tumor cells can be divided into two parts. One is to induce ferroptosis by inhibiting the Xct/GSH/GPX-4 pathway axis, leading to the accumulation of lipid reactive oxygen species, and the other is to directly or indirectly induce ferroptosis around iron metabolism and mitochondria (27, 29).

With the advantage that neoantigens are specific to tumor cells, it is possible to cause tumor cells to undergo pyroptosis and ferroptosis or to change the tumor microenvironment and cause tumor cell death by targeting genetic mutations. Controlling and intervening in ferroptosis can delay or stifle the occurrence and development of tumors, improve the prognosis of patients with tumors and prolong survival, suggesting the value of relevant studies.

TUMOR NEOANTIGENS AND PYROPTOSIS

Pyroptosis plays an important role in many diseases, and it also has a certain role in the treatment of cancer. Recently, there have been few studies in related areas, but studies promoting or inhibiting the occurrence of pyroptosis to prevent the occurrence and development of tumors are warranted.

The emergence of high-frequency irreversible electroporation (H-FIRE) provides a new method for the treatment of tumors (30). H-FIRE can effectively ablate the primary tumor and induce the pro-inflammatory metastasis of the tumor microenvironment (31). The most important thing is, H-FIRE can produce tumor neoantigens; for example, H-FIRE produces 4T1 neoantigens in the treatment of breast cancer, which activates the adaptive immune system and significantly reduces tumor progression (31). H-FIRE induces acute inflammatory response through various pathways, an essential mechanism of antitumor immunity (32). Some studies show that in treating pancreatic cancer, H-FIRE combined with PD-1/PD-L1 shows that H-FIRE ablation is superior to thermal ablation and cryoablation in inducing T cell immunity, suggesting that H-FIRE ablation combined with immunotherapy may play a synergistic role. H-fire could directly punch holes in cells to induce K^+ outflow and induce pyroptosis (32). It is also found that even if a small number of tumor cells pyroptosis after treatment, they could chemotactic $CD8^+$ T lymphocytes to accumulate in the tumor, thus effectively inhibiting tumor metastasis. Besides the stress state, the cells could induce pyroptosis through different signaling pathways (33, 34). Studies have found that *in vivo* and *in vitro*, the neoantigens produced by H-FIRE can interact with necrosis and pyroptosis-related cell death mechanisms through damage-related molecular signaling to kill tumor cells. Among these mechanisms, the pyroptosis of tumor cells may be due to the activation of NOD-like receptor (NLRS), absent in melanoma 2 (AIM2), etc., by noninfectious stimuli such as damage-associated molecular patterns (DAMPs). These events activate caspase-1, convert GSDMD to GSDMD-N, and promote the maturation and release of IL-1β and IL-18, causing pyroptosis to occur (35, 36). Studies show that STING (stimulator of interferon genes) agonists can activate NLRP3 by the caspase-1 pathway, thereby promoting the occurrence of pyroptosis and inhibit tumor progression in the Lewis mouse lung cancer model with neoantigen (37, 38). Therefore, it is speculated that STING agonists may inhibit the tumor by promoting pyroptosis of Lewis lung cancer cells with neoantigen.

In addition, a study found that retinoic acid can induce pyroptosis when activated by retinoic acid-inducible gene I

(RIG-1) (39). RIG-1 is a receptor that recognizes abnormal viral mRNA in cells and is a member of the DexD/H box RNA helicase family (40). The C-terminus of RIG-1 contains the unwinding domain, which can interact with artificially synthesized double-stranded RNA and viral double-stranded RNA and unwind it in an ATPase-dependent manner. The N-terminus contains two sequential caspase activation and recruitment domains (CARDs). Once RIG-1 is activated, it can exchange its CARD for the CARD of the inflammatory molecule, causing the cell to rupture and activating the key molecule caspase-1, which in turn can cause maturation of the proinflammatory factors IL-1β and IL-18. The cleavage of the executor protein GSDMD, which also activates pyroptosis, into GSDMD-N leads to the occurrence of pyroptosis (41–43). Some studies have shown that in the cancer environment, RIG-1 signaling in tumor cells can affect the complexity of the TME (44); RIG-1 activation can provide many benefits for the treatment of tumors: 1, directly killing tumor cells; 2. activating innate immune effectors such as macrophages and natural killer cells through cytokines; and 3. enhancing the activity of professional antigen-presenting cells (APCs), such as dendritic cells (DCs) or macrophages by making the microenvironment rich in cytokines and increasing adaptive immunity by recruiting relevant cells (such as CD8+ T lymphocytes). Therefore, research on RIG-1 agonists is of extraordinary significance for the treatment of various cancers (45–48).

In summary, promoting pyroptosis could further activate the innate immune system, inhibit the development of tumor cells by changing the TME, and even directly kill tumor cells. Study shows that medical chemotherapy can activate the pyrodeath signal and induce pyroptosis. Furthermore, chemotherapy drugs can activate GSDME, a tumor candidate, to promote pyroptosis. This study provides a theoretical basis for chemotherapy combined with immune checkpoint therapy (16). Therefore, pyroptosis is a promising potential target for the treatment of cancer.

TUMOR NEOANTIGENS AND FERROPTOSIS

Ferroptosis is known to lead to the accumulation of ROS. In normal cells, the high accumulation of ROS is detrimental to the cell. However, tumor cells are a unique case. Studies have shown that intracellular ROS accumulation has both advantages and disadvantages for tumor progression (49). Compared with normal cells, tumor cells show significant upregulation of ROS. Therefore, to maintain REDOX homeostasis, tumor cells have evolved a powerful ROS scavenging system. This dynamic mechanism to ensure homeostasis enables ROS to act as promoters for tumor development and progression. Therefore, ROS induction therapy or antioxidant inhibitor therapy can modify ROS levels to induce tumor cell killing, which is a new strategy for the effective and selective killing of cancer cells (50).

Research shows that some tumor cells undergoing drug-induced death are very sensitive to ferroptosis, so ferroptosis has become a popular topic in tumor therapy research in recent years. For example, dihydroartemisinin can induce ferroptosis in head and neck squamous cell carcinoma cells (51), artemisinin (ART) can induce ferroptosis in pancreatic cancer cells by specifically inducing ROS production (52), and siramesine and lapatinib can induce ferroptosis in breast cancer cells (53). Therefore, inducing ferroptosis of tumor cells to inhibit tumor proliferation and metastasis may become a new strategy for tumor therapy in the future. A study showed that the cystine/glutamate antiporter SLC7A11 (commonly known as XCT), which is overexpressed in a variety of human cancers, could be used for GSH biosynthesis and antioxidant defense. By inhibiting cell ferroptosis, the overexpression of SLC7A11 promotes tumor growth. However, tumor cells with high SLC7A11 expression also undergo metabolic reprogramming associated with SLC7A11 overexpression, leading to the dependence of SLC7A11-overexpressing cancer cells on glucose and Gln, which provides a potential metabolic target for the therapeutic targeting of SLC7A11 in cancer (54). Erastin is a compound that has a strong inhibitory effect on cancers that express the RAS gene, and it can mediate ferroptosis (7). Therefore, developing strategies that can improve the content, solubility and potency of erastin is of great significance for cancer treatment (55–57). Nanoparticle-induced ferroptosis has also been demonstrated in xenotransplantation studies (58). It was confirmed as early as 15 years ago that the tumor suppressor gene p53 can inhibit SLC7A11 and, in some cases, can also induce ferroptosis (59). In addition, studies have confirmed that the nuclear transcription factor NRF2 can inhibit the occurrence of ferroptosis in tumor cells by regulating the transcription of SLC7A11, and inhibiting the expression of NRF2 can enhance cell ferroptosis (60). More interestingly, CD8+ cells that can recognize specific tumor antigens (neoantigens) and are involved in antitumor activities can secrete high levels of interferon-γ (IFN-γ), which is a cytokine that is important for CD8+ T cells to complete their immune killing function (61). Studies show that tumor neoantigens promote tumor cell killing by CD8+ cells (62). In addition, IFN-γ can downregulate the expression of two Glu-cystine antiporter subunits, recombinant solute carrier family 3, member 2 (SLC3A2) and SLC7A11, on the surface of tumor cells. Therefore, IFN-γ inhibits the uptake of cystine by tumor cells, reduces the synthesis of GSH in the cell, and ultimately leads to the insufficient synthesis of GPX4 in the cell, thus preventing the effective removal of lipid peroxides. Ion-dependent conditions lead to ferroptosis of cells (27, 63, 64). As mentioned above, tumor-specific therapy can stimulate CD8+ cells to release IFN-γ to induce ferroptosis of tumor cells without causing injury to normal cells.

In addition, molecules related to the NF2/YAP (neurofibromin 2/Yes-associated protein 1) signaling pathway, which are often malignant mutations in cancer, have also been found to play an important role in the regulation of ferroptosis. Therefore, malignant mutation events in molecules involved in the NF2/YAP signaling pathway could predict the sensitivity of cancer cells to iron-induced death therapy (65). Malignant mutations related to the NF2/YAP signaling pathway could

also express related neoantigens on tumor cells. Therefore, in the future, it would apply the iron-chelating agents and antioxidants or SystemXC-inhibitors and GPXS inhibitors to regulate ferroptosis of tumor cells for malignant mutations related to the NF2/YAP signaling pathway. NF2/YAP signaling may be a potential target for the treatment of tumor cells.

APPLICATION OF TUMOR NEOANTIGENS

Advances in gene sequencing technology have enabled more tumor-specific immune targets to be discovered. In addition, tumor neoantigens that are only expressed in tumor cells can be recognized by the immune system, indicating that tumor neoantigens have a unique and important role in immunotherapy and warranting further development. Neoantigen-targeted tumor immunotherapy has already been actively attempted in the clinic.

The CAR-T and TCR-T technology

Tumor neoantigen-based immunotherapy has enabled precise cellular immunotherapy for human tumors (66) and suggests the possibility of individualized treatment of cancer patients. T cells are lymphocytes that mainly mediate antitumor immunity, so strategies using T cells to specifically recognize tumor neoantigens to kill tumor cells are rational. Chimeric antigen receptor T cell (CAR-T) technology is an individual immunotherapy technology based on neoantigens. It is a tumor therapy strategy in which patients T cells are extracted and T cells with chimeric antigen receptors (CARs) with strong affinity for tumor cells are selected, ultimately enabling binding and killing of tumor cells. CAR-T cells are currently mainly used for hematological cancers. At present, CAR-T cells have been approved for the treatment of various CD19-positive hematological malignancies (67). The extraction of T cells from patients for CAR-T development is the component that makes CAR-T technology expensive. If allogeneic CAR-T cell treatment can be realized, it will have extraordinary significance for the treatment of solid tumors. Such treatment can overcome the challenge related to the release of excessive proinflammatory factors by T cells during tumor cell killing. Allogeneic CAR-T technologies are already in clinical trials, including CRISPR-based strategies to edit T cells. If the utility of allogeneic CAR-T treatment can be clinically verified, modified CAR-T can be produced on a large scale, which may reduce costs. However, studies have found that CAR-T therapy also exhibits "off-target" effects. In the treatment of colon cancer patients, the targeting of V-erb-b2 avian erythroblastic leukemia viral onco-gene homolog 2 (ErbB2) by CAR-T technology unexpectedly led to a large number of CAR-T cells in the lungs of patients, which resulted in cytokine release syndrome (CRS) and multiple organ failure (MOF) (68). The preparation of CAR-T targeting tumor-specific neoantigens may solve the problem of "off-target" effects. For now, clinical trials of CAR-T cells targeting ErbB2, disialoganglioside (GD2), and prostate-specific membrane antigen (PSMA) as new targets are also underway (69).

The T cell antigen receptor (TCR) is a marker of all T cells and can be used to identify histocompatibility complex (MHC) molecules and antigen peptide complexes carried by antigen-presenting cells (70). At present, the TCR is the only known molecule that can sensitively recognize epitopes on the surface of target cells. This unique ability also establishes the pivotal role of the TCR in T cell transfection technologies.

It has been found that modified TCRs have several advantages compared with CARs. CARs can recognize antigens in the molar range, and the target density required for the reaction needs to reach $>10^3$ moles of antigen/cell. However, CARs can recognize peptides from the surface and intracellular proteins. They can also identify antigens in the micromolar range, and the target density required for the reaction only needs 1-50 moles of antigen/cell (71). Studies have shown that less than 25% of human proteins are membrane bound, and the proportion of amino acid sequences available on the cell surface (perhaps <10%) is low. Therefore, the number of antigens suitable for TCR targeting is much higher than that for CAR targeting (72, 73). CARs include a specificity determining region antibody fragment (single-chain antibody scFv variable region) that binds to specific antigens, a transmembrane domain (hinge/spacer domain, mostly from CD8) and multiple signal transduction elements (such as sequences from CD28, 4-1BB/OX40, CD3ζ and other important T cell molecules) (74–76); these domains determine the specificity of the CAR: surface proteins, glycoproteins, glycolipids, carbohydrates, or other antigens. However, the TCR recognizes the peptide-major histocompatibility complex (pMHC) complex inside and outside the cell. Therefore, these advantages are particularly important for the treatment of solid tumors. TCR targeting of tumor cells is an important strategy.

After the efficacy of CAR-T and TCR-T in treating hematologic malignancies, attention has shifted to CAR-engineered NK (CAR-NK) and TCR-engineered NK (NK-TCR) engineering. NK is a natural killer cell that has a strongly antitumor ability in the immune system. NK-TCR could improve the responsiveness and recognition specificity of NK cells to tumor cells (77). And CAR-NK has better safety and multiple mechanisms to activate cytotoxicity. NK cells also have a low risk of graft and host disease (GVHD) to be prepared in advance for use in multiple patients. CAR-NK cells can be designed to target multiple antigens, enhance proliferation and *in vivo* persistence, increase invasion of solid tumors, overcome drug-resistant tumor microenvironments, and ultimately achieve an effective antitumor response (78).

In summary, CAR-T and TCR-T technologies have similarities in the treatment of malignant tumors and are rapidly advancing, but ACT is the most promising treatment. The most important goal that must be accomplished for CAR-T or TCR-T technology to cure malignant tumors is identifying suitable target antigens. Although NK-CAR and NK-TCR technologies are not immature and have some technical and clinical challenges, their emergence offers more options and broad therapeutic prospects for cancer treatment.

Anti-PD-1/PD-L1 Antibody Therapy

The programmed death receptor (PD-1) and its programmed death protein receptor-ligand (PD-L1) are called immune checkpoints, and they are important proteins for immune regulation. One study found that agents blocking cytotoxic T cell antigen 4 (CTLA-4) in combination with PD-1/PDL-1 blockade induced tumor regression in a portion of patients (79). This method is mainly aimed at activating the adaptive immune system in tumors with restricted checkpoint inhibition (80). Tumors with more mutations are likely to produce more new epitopes. These epitopes can be recognized by tumor-infiltrating T cells, but antibodies that block the checkpoint lead to tumor-infiltrating T cell activation and cause tumors to regress, so highly mutated tumors such as melanoma and lung cancer are more sensitive to anti-PD-1/PD-L1 antibody therapy (81, 82).

The first and most studied biomarker is PD-L1 protein expression. It is a potential biomarker of anti-PD-1/PD-L1 drug response. The clinical utility of immunohistochemistry (IHC) for detecting the expression of PD-L1 on tumor cells and/or tumor-infiltrating immune cells was confirmed initially in the first clinical study of the anti-PD-1 drug nivolumab and subsequently in other studies. The mechanisms of PD-L1 have been deeply studied (83).

The expression of PD-L1 is also closely related to prognosis. Some studies have collected information about PD-L1 overexpression in patients with gastric cancer, hepatocellular carcinoma, esophageal cancer, pancreatic cancer, ovarian cancer, and bladder cancer. PD-L1 expression has been shown to have an impact on prognosis in breast cancer and Merkel cell carcinoma patients, but the prognostic implications of PD-L1 expression are controversial for patients with lung cancer, colon cancer, and melanoma (84). For example, in kidney cancer, studies have found that the higher the expression of PD-L1 is, the more advanced the tumor stage and the worse the prognosis (85). Similarly, high expression of PD-L1 has a negative relationship with the survival rate of patients with non-small-cell lung cancer, but this idea has not been supported by clear evidence (86).

Cancer Vaccines

The ideal tumor vaccine can efficiently induce humoral immunity and cellular immunity. While it is important to induce the proliferation and activation of specific T cells to enhance the killing of tumor cells *in vivo*, it is also necessary to prevent tumor vaccines from inducing recognition of TAAs that would trigger an autoimmune response. New antigen vaccines mainly include DC vaccines, peptide vaccines, DNA/RNA vaccines, antibody tumor vaccines, etc.

Because DCs are professional APCs, they can be used as effective inducers of tumor-specific immune responses (87). DC vaccines activate the immune functions of CD8 and CD4 T lymphocytes by loading tumor peptides and eliminate tumor cells. It was found that DCs can not only activate T cells but also maintain a balance between immune activation, suppression, and memory (88, 89). It is well known that DCs also recognize pathogen-related molecular patterns (PAMPs) and DAMPs through pattern recognition receptors (PRRs) (88), and pyroptosis is also induced by these molecular patterns. Strategies to inhibit the occurrence of DC pyroptosis, which could increase the number and quality of DCs to further kill and eliminate cancer cells, are worth considering.

Antigen polypeptides eluted from the surface of tumor cells can be used to generate polypeptide vaccines, which direct immune cells to target abnormally expressed proteins inside tumor cells with strong specificity and safety. At the same time, further modification of amino acid residues or preparation of heat shock protein-peptide complexes can not only effectively improve the specificity of polypeptide antigens but also avoid autoimmunity directed at host cells. Studies have found that tumor peptide vaccines can control perihepatic tumor lymph node metastases (pLNMs), improve cancer prognosis, and increase the survival rate. The literature shows that combination of peptide vaccines with immune checkpoint inhibitor therapy may induce a synergistic effect to further improve the effects of peptide vaccines in clinical treatment (90) (**Figure 4**).

For example, tumor vaccine strategies have advanced, and tumor vaccines can be made according to individual differences. The development of such tumor vaccines has laid the foundation for individualized tumor treatment. DNA sequencing technology can be used to identify tumor-specific mutations. The sequence of a new antigen can be quickly inferred to make a vaccine. Once the cancer vaccine induces activation of specific T cells, anti-PD-1/PD-L1 antibodies can be used to enhance these immune responses against cancer cells (91). However, there are still many technical problems to be solved regarding tumor vaccines. For example, the vaccine preparation cycle is too long. In addition, it is necessary to sequence the genome of the patient's tumor cells to identify relevant sequences to prepare the vaccine. Such a cycle may be too long for some advanced tumor patients. In addition, the cost makes this technology impractical.

DISCUSSION

As mentioned above, pyroptosis and ferroptosis have many effects on the occurrence, development, treatment, and survival of tumors. Pyroptosis and ferroptosis can kill tumor cells through different mechanisms. Therefore, these methods of PCD inhibit the occurrence and development of tumors and improve the survival rate of patients. Pyroptosis can also modify the anti-inflammatory tumor microenvironment *in situ* in a variety of ways to inhibit the growth and survival of tumor cells and can enhance the ability of APCs such as DCs and macrophages to activate the innate and adaptive immune systems. Pyroptosis can also enhance the recruitment of CD8+ T lymphocytes. The exchange of CARDs between RIG-1 and inflammatory molecules leads to the occurrence of tumor cell pyroptosis and delays the development of tumors. Research on the role of ferroptosis in the treatment of tumors has become more extensive. Studies have found that, on the one hand, tumor cell ferroptosis can be controlled, for example, by combined

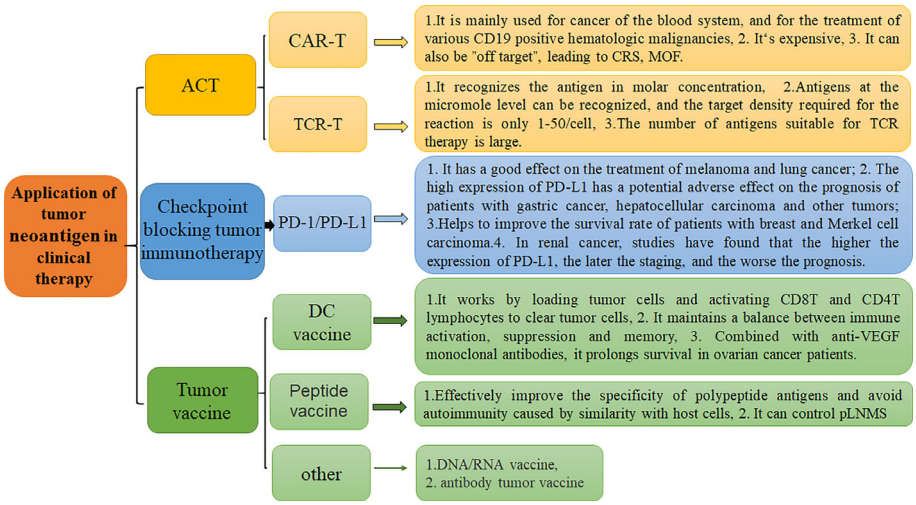

FIGURE 4 | Application of tumor neoantigen-based strategies in clinical therapy and their respective characteristics. There are three primary clinical treatment methods, including ACT, Checkpoint blocking tumor immunotherapy, and tumor vaccine.

application of ROS inducers to regulate the level of ROS to treat pancreatic cell carcinoma (92). On the other hand, many drugs are able to induce ferroptosis of tumor cells.

Although the mechanism is not clear, they are likely to become new agents for future tumor treatments. However, the research on pyroptosis and ferroptosis in tumors is still not sufficient, and clinical applications are lacking, especially strategies including the combined targeting of pyroptosis, ferroptosis and tumor neoantigens for the treatment of cancer; such strategies are worthy of further investigation. For example, STING agonists could trigger pyroptosis, especially in Lewis mouse lung cancer cells with neoantigens. It may be due to the activation of caspase-1/NLRP3/GSDMD related pathway molecules by sting agonists. Simultaneously, the tumor cells of the Lewis mouse lung cancer model are more sensitive to sting agonists, leading to tumor pyroptosis. We can take advantage of this feature. It can make lung cancer cells pyroptosis and inhibit tumor progression.

Meanwhile, NF2/YAP is an essential molecular signal that regulates ferroptosis and is also a gene responsible for malignant mutations found in cancer. It is inferred that neoantigens produced by NF2/YAP mutated cancer cells can be identified by serological analysis techniques of recombinant expression cDNA clones, total exon sequencing (WES) combined with RNA sequencing (RNA-seq) and epitope prediction methods, and LC-MS/MS methods. To regulate NF2/YAP molecular signal to promote cancer cell ferroptosis, thereby inhibiting tumor occurrence and development and improving survival rate is possible. So using tumor cell neoantigens to target tumor cells and cause pyroptosis or ferroptosis is an important strategy for the future.

The accuracy of neoantigen screening methods needs to be improved, and the techniques need to be simplified to enable the development of neoantigen-based immunotherapies. Of course, reducing costs is also very important. Immunotherapy strategies related to neoantigens are rapidly being developed. The individualization of cancer treatment, prevention of treatment side effects, timeliness of treatment, and reduction of treatment costs are remaining challenges.

AUTHOR CONTRIBUTIONS

JY and QW, Writing-original draft. XC and ZG, Writing, review and editing. XZ and XC, Funding acquisition. All authors contributed to the article and approved the submitted version.

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REVIEW
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Safety and Efficacy of Personalized Cancer Vaccines in Combination With Immune Checkpoint Inhibitors in Cancer Treatment

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Cancer immunotherapy can induce sustained responses in patients with cancers in a broad range of tissues, however, these treatments require the optimized combined therapeutic strategies. Despite immune checkpoint inhibitors (ICIs) have lasting clinical benefit, researchers are trying to combine them with other treatment modalities, and among them the combination with personalized cancer vaccines is attractive. Neoantigens, arising from mutations in cancer cells, can elicit strong immune response without central tolerance and out-target effects, which is a truly personalized method. Growing studies show that the combination can elevate the antitumor efficacy with acceptable safety and minimal additional toxicity compared with single agent vaccine or ICI. Herein, we have searched these preclinical and clinical trials and summarized safety and efficacy of personalized cancer vaccines combined with ICIs in several malignancies. Meanwhile, we discuss the rationale of the combination and future challenges.

Keywords: personalized cancer vaccine, immune checkpoint inhibitor, combination therapy, neoantigen, immunotherapy

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INTRODUCTION

Neoantigen, an abnormal protein stemming from "non-synonymous mutation", is specific to tumor cells (1). Neoantigens are non-self-peptides without central tolerance and off-target immune-toxicity, which are the main barriers of previous cancer vaccines and the primary obstacles in the development of personalized cancer therapy (2–4). Therefore, they are "perfect" targets with strong immunogenicity to elicit effective antitumor activity. Currently, a lot of preclinical and clinical trials have proven that neoantigen vaccines are personalized therapy and can activate hosts' immune systems which then promote redirected T cells to kill tumor cells. However, the single use of neoantigen vaccines has a limited efficacy.

In 2013, immune therapy of cancer was regarded as the most breakthrough in Science. From then, immunotherapy became a major focus in cancer therapy, of which immune checkpoint inhibitors are the

Abbreviations: CRT, calreticulin; ATP, adenosine triphosphate; HMGB1, High Mobility Group Box 1; HSPs, heat shock proteins; LN, lymph node; DCs, dendritic cells; CTLs, cytotoxic T lymphocytes; IDO, indoleamine 2,3-dioxygenase; mAb, monoclonal antibody; LAG3, Lymphocyte activation gene 3; BTLA, B and T lymphocyte associated gene.

most promising and concerned topic (5). Immune checkpoint inhibitors such as anti-PD-1 and anti-CTLA-4 monoclonal antibodies, improve antitumor efficacy and prolong overall survival time in patients with many solid tumors, including lung cancer, melanoma, gastrointestinal cancer and so on (6–9). Though immune checkpoint inhibitors mark the arrival of a new era of cancer immunotherapy, using them alone has limited effect, for many patients encounter primary resistance or initial responses but eventually becoming resistant (10–12). Therefore, there is an urgent need to combine other treatments with ICIs to improve the therapeutic efficacy and prolong overall survival.

Immune checkpoint inhibitors produce antitumor effects through eliminating immune inhibition, recovering or even enhancing hosts' immunity. The process of immune responses includes capturing, presenting, recognizing targets on tumor cells and finally killing tumor cells. Among so many targets on tumor cells, neoantigens are ideal ones to activate the immune system. Moreover, the neoantigen-specific CD8+ T cell reactivity plays a core role in immunotherapy (13). With observations that the absence of pre-existing immunity or the inhibition of tumor microenvironment may lead to invalidation of both methods (10, 14, 15), there is a strong rationale for combining ICIs with neoantigen vaccines (16). On one hand, adding neoantigen vaccine to ICIs can improve response rates of "hot" tumors through broadening cytotoxic T cell repertoire, as well as turn "cold" tumors to "hot" ones, therefore widening the scope of population who can benefit from immunotherapy (17–26). On the other hand, ICIs can unleash immunity to facilitate the efficacy of neoantigen vaccine. In this review, we focus on the safety and efficacy of personalized cancer vaccines combined with ICIs for the treatment of several malignancies. We highlight the recent development, challenges and possible improvements of personalized cancer vaccines in combination with ICIs, and hope to provide theoretical foundations for the development and application of personalized cancer vaccines in clinical settings.

RATIONALE FOR COMBINATION IMMUNOTHERAPY

To better understand the mechanism of combination immunotherapy, it is necessary to learn the dynamics of anti-tumor immune responses. Researchers propose a concept called "Cancer-Immunity Cycle", which illustrate crucial points during anti-tumor response and consist of seven steps (27). First step, dying tumor cells release tumor antigens such as CRT, HSPs, HMGB1 and ATP. Insufficient tumor antigens release may hamper the proceeding of this cycle. Second step, immature dendritic cells (DC) capture these antigens *via* signals such as CD92, TLR4 and P2RX7, which can bind to CRT, HMGB1 and ATP, respectively. Then the DCs maturate and migrate to draining lymph nodes. Inadequate activation of DCs may halt the process. Third step, DCs will process the captured tumor antigens and present them to prime and activate effector T cells. In this process, captured antigens with MHC class I and II molecules and DC co-stimulatory signals are required to stimulate T cells. However, some factors may affect T cell priming and activation, including defective expression of MHC molecules in tumors, over-expression of inhibitory signals (CTLA4/CD80, 86, PD-1/PD-L1), limited T cell repertoire (central tolerance), suppressive cells such as regulatory T cells (Tregs). Fourth and fifth steps, the activated effector T cells traffic to and infiltrate into tumors. Sixth and final steps, these cytotoxic T lymphocytes recognize *via* MHC/peptide complexes on the cell surface and kill their target cells. Many inhibitory mechanisms are active within tumor microenvironment, such as lack of MHC molecules in tumors, increased inhibitory signals (PD-1/PD-L1, Tim-3/phospholipids, BTLA, LAG3, IDO, Arginase), Tregs, myeloid-derived suppressor cells, M2 macrophages and hypoxia. When eventually killing tumor cells, they also release additional tumor antigens to provoke further Cancer-Immunity Cycle. This secondary immunity increasing the repertoire of tumor antigens is recognized as "antigen spreading" with increased breadth and depth of anti-tumor immunity (28). However, tumors are clever, which can take various strategies to attenuate the efficiency of anti-tumor immunity, resulting in incompetent immunity in a process designated as "cancer immune-editing" (29, 30). Therefore, the Cancer-Immunity Cycle is broken. Given the complicated immunity network, it is reasonable to combine appropriate immunotherapeutic strategies to pave the way and promote the Cancer-Immunity Cycle forward. For instance, neoantigen vaccines can bypass the first two steps and directly initiate an immune cycle. Immune checkpoint inhibitors can help overcome immune-suppression in steps 3 and 6. On the basis of cancer immunity cycle and recent studies (25, 31–38), we conclude the main mechanisms for combination immunotherapy are as follows.

Improve Sensitivity and Efficacy of Immune Checkpoint Inhibitors

Many patients with cancer initially do not response to anti-PD-1 inhibitors, possibly because of "cold" tumors, which are with no or few immune cells in tumor tissues and insensitive to ICIs (10, 39–41). In these tumors, tumor antigen cannot effectively prime and activate T cells, and further lead to the cycle halting at step 1 or 2. Vaccination with neoantigens bypassing initial two steps can produce many neoepitope-specific T cells which can traffic to tumor microenvironment and destroy tumor cells expressing these antigens. For instance, neoantigen-specific T cells were found in periphery blood after vaccination (10, 42–44). Furthermore, this strategy produces CD8+ neoantigen-specific T cells and memory T cells, and broadens the TCR repertoire of T cells, intensifying steps 3 and 4, which can further lead to tumor regression (16). Meanwhile, other studies have shown that clonally expanding neoantigen-reactive cells within tumor infiltrating lymphocytes (TILs) expressed PD-1 orPD-L1, suggesting that neoantigen vaccines could create a proper setting for ICIs and lay a foundation for the combination (31, 45). In conclusion, a potent personalized cancer vaccine with strong immunogenicity, can diversify the tumor-specific T cell repertoire, activate immune systems, activate robust effective T cells responses, and enhance the efficacy of ICIs.

Overcome Acquired Resistance of Immune Checkpoint Inhibitors

Effective ICIs result in immune-editing, which will result in subpopulation change, depletion of neoantigens, T cells expansion constraint and finally resistance to ICIs (46–49). Neoantigen repertoire variation can also lead to acquired resistance to ICIs (50). Immune-editing and variation of neoantigen repertoire are involved in many steps of the immunity cycle. In a study, patients with non-small cell lung cancer, who became resistant to ICIs after initial response, experienced the evolution of tumor neoantigens. Neoantigens, which were targets of initial response to ICIs, were eliminated in this process (50). Meanwhile, another study showed that loss of neoantigens could deter the specific T cells expansion (51). However, in the study, two patients with recurrent tumor lesions and resistant to ipilimumab, had tumor regression after injecting neoantigen vaccines (52). Researchers have tried to explore the influence of neoantigen cancer vaccines on neoantigen-specific T cell receptor repertoire, and they found that vaccination with neoantigens could elevate TCR-β clonotypes (52). What's more, neoantigen vaccines, which can provide strong immunogenicity to activate immune system based on the neoantigen variation spectrum, eventually overcome the acquired resistance (50). Therefore, identifying neoantigens in the tumor evolution and listing them as targets of personalized cancer vaccines can improve antitumor efficacy of ICIs in patient with resistance. Neoantigen vaccines not only speed the proceeding of the immunity cycle, but also augment some crucial points in the activity such as steps 3 and 4.

Immune Checkpoint Inhibitors Overcome Suppressive Microenvironment

The suppression of tumor immune microenvironment is the main reason for the failure of neoantigen vaccine alone to control tumor. Some researchers found that after the neoantigen vaccine was applied, the expression of PD-1 on neoantigen-specific T cells and PD-L1 on tumor cells increased. Meanwhile, compared to CD8+ TILs, neoantigen-specific TILs displayed a more exhausted-like phenotype, which indicated that neoantigen vaccines could contribute to the inhibitory immune microenvironment (1, 13, 53–55). Both conditions weaken the potency of steps 3, 6 and 7. Some researchers tried to investigate the effectiveness of ICIs to overcome the immune-suppression and prove that ICIs could diminish the suppression of immune system and help induce strong T cells targeting at neoantigen epitopes (18). Schumacher and colleagues conducted some studies and indicated that adding ICIs to the treatment of neoantigen vaccine could elevate neoantigen T-cell response (18). In another study, in one out of three melanoma patients who have a relapse and distant metastasis after vaccination with neoantigens, a complete response was observed by subsequent pembrolizumab treatment (53). ICIs can mitigate the impact of inhibitory factors such as PD-1, PD-L1 and CTLA-4 to the Cancer-Immunity Cycle. The general hypothesis is that ICIs may unleash neoantigens with less immunogenicity or reactivate T cells with exhausting phenotypes to enhance antitumor effects (13, 24, 56–59). Increasing studies certify that ICIs can relieve immune inhibition in neoantigen vaccine, and have a promising prospect (50, 60).

PRECLINICAL AND CLINICAL TRIALS AND RECENT DEVELOPMENT

A personalized cancer vaccine, when combined with the ICIs, has shown efficacy in many preclinical trials. Meanwhile, there are many finished and on-going clinical trials trying to further prove the efficacy of the combination in real world.

Recent Preclinical Trials of the Combinatory Modality

In the aggressive glioblastoma CT2A murine model, researchers generated the neoantigen vaccine comprising 27-mer peptides targeting the mutant Plin2G332R, Pomgnt1R497L, and Epb4H471L neoepitopes, as well as poly-ICLC adjuvant. Mice treated either with vaccine or anti-PD-L1 alone exhibited a median overall survival of 17.5 and 25 days, respectively. In contrast, 60% of mice treated with vaccine and anti-PD-L1 blockade demonstrated long-term survival. What's more, tumor-infiltrating neoepitope-specific CD8 T cells increased in the combinatory condition (25). In five murine colon carcinoma models, researchers investigated the clinical efficacy of the combining a peptide or DNA vaccine with anti-PD-1/L1 and anti-CTLA-4. The neoantigen vaccines combined with immune checkpoint inhibitors can potentiate neoantigen-specific immunity, elicit robust and long-lived T-cell response with a more diversified TCR repertoire and potentially inhibit even eradicate tumors without re-challenge (16, 49, 61–63). Furthermore, three studies have initially proven the central role of CD8+ T cells in the combination regimen by depletion of CD8+ T cells, CD4+ T cells and natural killer 1.1, respectively (16, 49, 63). Besides, vaccines with nanocomplexes markedly improve Ag/adjuvant co-delivery to lymphoid organs and sustain Ag presentation on dendritic cells (61–64). In two highly aggressive and poorly immunogenic murine models of B16F10 melanoma, researchers obtain the similar result that neoantigen vaccine combined with ICIs can initiate potent anti-tumor efficacy (62, 64). Additionally, Kuai et al., also reported vaccines administered *via* the subcutaneous (SC) or intramuscular (IM) routes were well tolerated in mice without any significant systemic or local toxicity, whereas SC could more efficiently deliver vaccines and intensify neoantigen-specific T cells responses (64). Finally, Panc02 cells models provide proof of concept that triple therapy with PancVAX (a personalized cancer vaccine), anti-PD-1, and agonist OX40 induces vaccine-specific TILs, lower the threshold for T cell activation, and reducing TIL exhaustion markers such as LAG3 and PD-1. In KPC mice (with KRAS and p53 mutations, pancreatic ductal adenocarcinoma), a "cold" tumor, combination treatment can also elicit objective tumor responses and prolonged survival. More importantly, this study shows that sequential combination treatment, neoantigen vaccine prior to anti-PD-1 antibodies significantly increased

INF-γ expression and cure rates compared to single regimen or in combination with anti-PD-1 blockadeconcurrently (65, 66). What's more, in a hepatic cell cancer model, similar results are reported (67). These preclinical trials are displayed in **Table 1**.

Recent Completed and Ongoing Clinical Trials

The first open-label phase IB clinical trial (NCT02897765) of a personalized neoantigen-based vaccine, NEO-PV-01, in combination with PD-1 blockade, included 82 patients with advanced solid tumors. Analyzing 82 patients, the median progress free survival (PFS) among vaccinated patients was 23.5, 8.5, and 5.8 months in the melanoma, NSCLC, and bladder cancer cohorts, respectively. The median OS for vaccinated patients was not reached in the melanoma and NSCLC cohorts, while for the bladder cancer cohort, the median OS was 20.7 months. The primary objective of the study was to evaluate the safety and tolerability of NEO-PV-01 in combination with nivolumab. The most common adverse events in vaccinated patients were injection-site reactions and influenza-like illness (52 and 35% of the patients, respectively). No treatment-related serious adverse events were observed. These data support the safety and immunity of this regimen in patients with advanced solid tumors (68).

A phase IB study (NCT03289962) evaluated RO7198457, an individualized neoantigen-specific Immunotherapy (iNeST), in combination with atezolizumab in 144 patients with locally advanced or metastatic solid tumors. RO7198457 is a kind of mRNA vaccine including up to 20 neoantigens. In 108 analyzable patients, nine patients responded to the therapy (ORR 8%) including one complete response (CR) and 54 patients (49%) experienced stable disease (SD). The vaccine induced neoantigen-specific T cell response in 77% patients. The combination was well tolerated, and most adverse events were infusion-related reaction, fatigue, cytokine release syndrome, nausea, pyrexia in over 10% patients, classified as grade 1 or 2. No increase in immune-mediated AEs compared with anti-PD-1 alone. Results prove that the combination of RO7198457and atezolizumab is safe and effective, which can induce significant neoantigen-specific immune response (69).

Another phase I multicenter study (NCT03313778) is to assess the safety, tolerability, and immunogenicity of mRNA-4157 alone in 13 patients with resected solid tumors and in combination with pembrolizumab in 20 patients with unresectable solid tumors. Indications include melanoma, NSCLC, MSI-high CRC, metastatic cutaneous squamous cell cancer, bladder cancer and so on. mRNA-4157, is a lipid-encapsulated RNA-based neoantigen-based vaccine. Of the 13 patients, 12 patients remain disease free on study with median follow-up of 8 months. While in another 20 patients, researchers observed one CR, two partial responses (PR), five SD, five progressive diseases (PD), two introduced immune unconfirmed progressive disease, and one patient non-evaluable for response. No dose of limited toxicity (DLT) and no drug related SAEs or AEs ≥ grade 3were reported, and treatment related AEs have generally been of low grade and reversible. These results also demonstrate the antitumor efficacy and safety of the combination with pembrolizumab and neoantigen-specific T cells, proceeding mRNA-4157 to phase2 (70, 71).

ADXS-NEO-02 is an ongoing Phase 1 trial (NCT03265080), which preliminarily investigates the safety and efficacy of ADXSNEO alone and in combination with anti-PD-1 antibody therapy in solid tumors. ADXS-NEO is composed of the

TABLE 1 | Preclinical trials of neoantigen vaccines combined with immune checkpoint inhibitors.

Type of neoantigen vaccine	Formulation	Type of tumor	Immune checkpoint inhibitor	Antitumor effects comparing to vaccine or ICIs alone	Route of vaccination	Ref
Peptide	Albumin/AlbiAdpgk nanocomplexes	Colon cancer(MC38 tumor)	Anti-PD-1	More effective	SC	(61)
Peptide	sHDL-Adpgk and adjuvants	Colon cancer and melanoma (MC-38 and B16F10 tumors)	Anti-PD-1, anti-CTLA-4	More effective	SC	(62)
Peptide	sHDL-Adpgk/CpG	Advanced B16F10 melanoma tumors	Anti-PD-1, anti-CTLA-4	More effective	SC or IM	(64)
Peptide	PancVAX	Pancreatic adenocarcinoma (Panc02 cells)	Anti–PD-1	More effective	SC	(65)
DNA	poly-neoantigen DNA vaccine	Colorectal tumor MC38	Anti-PD-1	More effective	Intradermal injection	(49)
DNA/mRNA	GAdCT26-31/GAd-MC38-7	Colon carcinoma (CT26 cell line, MC38 cell line)	Anti-PD1, anti-PDL1	More effective	SC	(16)
Peptide	Lm-ANXA2	Pancreatic carcinoma	Anti-PD1	More effective	Injection directly through the spleen	(66)
Peptide	Adpgk with adjuvants (banNVs)	Colorectal cancer	Anti-PD-1	More effective	SC	(63)
Peptide	Multivalent neoantigen vaccine	Glioblastoma (CT2A GBM model)	Anti–PD-L1	More effective	None	(25)
Peptide	Thiolated nano-vaccine	Hepatocellular carcinoma(H22 cells)	Anti–PD-1	More effective	SC	(67)

SC, subcutaneous; IM, intramuscular; GAdCT26-31/GAd-MC38-7, vaccines of great ape adenovirus encoding multiple neoantigens; Lm-ANXA2m listeria-based, ANXA2-targeting vaccines; Adpgk, ADP dependent glucokinase; CpG, cytidine-phosphate-guanosine; sHDL, synthetic high-density lipoprotein; PancVAX, neoantigen-targeted vaccine.

listeriolysin O(tLLO) and personalized tumor antigens with 20–21mer peptides. This initial result reveals the optimal dose of ADXS-NEO with safety and efficacy. Only mild/moderate, controlled, and reversible events (e.g., chills, fever, tachycardia) were observed at this level. Efficient and rapid priming of substantial CD8+ T cells against most neoantigens and increased secretion of chemokines consistent with T-cell trafficking into tumor microenvironment also existed. Further investigation results about the combination of ADXS-NEO + anti-PD-1 antibody therapy are waiting (72).

Another ongoing phase I/IIA study (NCT03633110), is exploring the tolerability and antitumor activity of GEN-009 combined with anti-PD-1/L1 in multiple advanced tumors. GEN-009 is a personalized neoantigen-based vaccine comprising 4–20 synthetic long peptides formulated with poly-ICLC. The preliminary study of GEN-009 alone in solid tumors displayed that neoantigen provoked sustained peripheral neoantigen-specific CD4+ T cell and CD8+responses in all eight patients. Besides, repeated dosing has been well tolerated with mild local discomfort and no DLT. Further data from this trial about the vaccine in combination with PD-1 inhibitors in patients with advanced tumors, are awaited (71, 73).

Another open-label, phase IB study (NCT03380871) of NEO-PV-01 with pembrolizumab plus chemotherapy in patients with advanced or metastatic non-squamous non-small cell lung cancer is completed, and the results are expected. More clinical trials combining personalized vaccines with immune checkpoint inhibitors are listed in **Table 2**.

SAFETY AND TOXICITY ASSOCIATED WITH THE COMBINATION MODALITY

The application of ICI therapy comes with the possibility of occurring immune related adverse events (IRAE). IRAE are regarded as an "over-activation" of the immune system leading to autoimmune inflammatory events affecting virtually any organ, most commonly the skin, gastrointestinal tract, liver, endocrine system and lung (74–76). In this review, we have highlighted the combination of neoantigen vaccine with ICI. Neoantigen vaccine and ICI both effect *via* provoking the immune system. Consequently, there is a relevant concern that the combination may lead to excessive toxicity. Overall, clinical experience with the combination strategies discussed in this review is limited. However, in a phase I trial (NCT02897765 n = 82) evaluating the safety and tolerability of NEO-PV-01 in combination with nivolumab, injection-site reactions (52%) and influenza-like illness (35%) are most common. Injection site reactions such as warmth and erythema, were often reversible and mild (Common Terminology Criteria for Adverse Events [CTCAE] grade 1), except one patient with a grade 2 injection site erythema. Drug-related events of grade ≥3 severity appeared in two patients. One patient with grade 2 gastritis discontinued the treatment. There is no treatment-related serious adverse events (68). In another phase Ib study (NCT03289962 n=142) to assess RO7198457 in combination with atezolizumab, immune-mediated adverse events (AEs) in atezolizumab alone were similar to those of the combination in >10% of patients include infusion-related reaction (60%), cytokine release syndrome (15%), influenza-like illness (10%), fatigue (30%), nausea (22%), pyrexia (15%), diarrhea (19%), decreased appetite (15%), vomiting (14%), headache (12%), cough (15%), dyspnea (15%), arthralgia (10%), constipation (15%),anemia (12%). Individual signs and symptoms of systemic reaction in more than five patients involve pyrexia, chills, nausea, tachycardia, headache, vomiting, hypertension, myalgia, back pain, fatigue, and hypoxia. 1% patients had grade 3 infusion-related reactions, anemia, fatigue, dyspnea, vomiting, nausea, pyrexia, diarrhea, headache, respectively. Most AEs are grade 1 or 2, and systemic reactions were transient and generally manageable in the outpatient setting. No grade 4 or 5 AE was observed (69). InmRNA-4157 trial, there was also no serious treatment related AEs, with only low grade and reversible reactions. Generally speaking, initial results of studies prove the treatment regimen of neoantigen vaccine combined with ICIs efficient and safe. Whereas, the actual risk for severe adverse events with combinations and potential factors influencing safety such as dose, delivery platforms, ways of administration, adjuvant, personal status, will be required to more and larger randomized studies.

MAIN CONSIDERATIONS RELATING TO THERAPEUTIC COMBINATORY REGIMEN

Various factors may affect the anti-tumor efficacy in the combinatory modality. For neoantigen vaccine, neoantigen selection is critical, which will determine the production of antigen-specific T cells and eventually influence the anti-tumor response. The pipeline for neoantigen identification includes five main steps, and details have been extensively reviewed in other reviews (77–79). Unfortunately, there is no perfect method to predict appropriate neoantigen without bias and negatively positive. What's more, the selection of proper delivery platforms is also crucial. Seven kinds of vectors are explored, including synthetic peptides, messenger RNA, DNA plasmids, viral vectors (adenoviral and vaccinia), engineered attenuated bacterial vectors (Salmonella, Listeria), *ex vivo* antigen-loaded DCs, and nanodiscs. Their advantages and disadvantages have been listed in detail in previous reviews (80, 81). The central problems are the delivery efficacy and manufacturing time, which play an important role in initiating enhanced T cell responses to inhibitor tumors. The sequence of manufacturing time from shorter to long is approximately tumor lysate-pulsed DCs, DNA/RNA, peptide, neoantigen-pulsed DC (71). For both peptide and mRNA vaccine platforms, time less than 4 weeks is expected (80). Additional variables include the route of administration of the vaccine, total number of doses, and induction (priming) and booster (maintenance) intervals. In a preclinical trial, Rui Kuai et al. declared that subcutaneous injection, comparing with intramuscular way, have stronger capability to deliver vaccines and provoke neoantigen-specific T cells responses (64). With the dose increasing, the immune response is intensified and the occurrence of immune related

TABLE 2 | Recent clinical trials combining neoantigen vaccines with immune checkpoint inhibitors.

ClinicalTrial.gov identifier	Phase	Enrollment status	Cancer Type	Neoantigen Formulation	Additional intervention	Delivery	Dose and Schedule
NCT04397003	II	Not yet recruiting	ES-SCLC	Neoantigen DNA	Anti-PD-L1 (durvalumabMEDI4736)	Intramuscular by electroporation	Not specified, six q4w cycles
NCT03897881	II	Recruiting	M	Neoantigen mRNA-4157	Anti-PD-1(pembrolizumab)	None	1,000 mg nine q3w cycles
NCT04267237	II	Withdrawn	NSCLC	Neoantigen mRNA (RO7198457)	Anti- PD-L1(atezolizumab)	Intravenous infusion	Not specified, 12 q4w cycles
NCT03815058	II	Recruiting	Advanced M	Neoantigen mRNA (RO7198457)	Anti-PD-1(pembrolizumab)	None	Not specified; qw priming and booster
NCT03606967	II	Recruiting	Metastatic TNBC	poly-ICLC+ Neoanitgen synthetic long peptides	Anti-PD-L1 (durvalumabMEDI4736)	Subcutaneous	None
NCT03598816	II	Withdrawn	RCC	Neoantigen DNA	anti-PD-L1 (durvalumab]) anti-CTLA-4 (tremelimumab)	Intramuscular	six doses
NCT03953235	I, II	Recruiting	NSCLC, CRC, PC, other solid tumors	Neoantigen Peptides (GRT-C903 and GRT-R904)	anti-PD-1(Nivolumab)and anti-CTLA-4(ipilimumab)	None	None
NCT03639714	I, II	Recruiting	NSCLC, MSSCRC, EC, BC	Neoantigen adenovirus vector + self-amplifying mRNA (GRT-C901 and GRT-R902)	anti-PD-1(nivolumab) and anti-CTLA-4(ipilimumab)	Intramuscular *via* viral vector	30–300 mg, Priming and booster
NCT04251117	I/IIA	Recruiting	HCC	Neoantigen DNA vaccine (GNOS-PV02)	Anti-PD-1 (pembrolizumabMK-3475)	Intradermal injection and electroporation	None
NCT03164772	I, II	Active, not recruiting	NSCLC	mRNA Vaccine (BI 1361849, CV9202)	anti-PD-L1 (durvalumab) + anti-CTLA-4 (tremelimumab)	None	None
NCT04024878	I	Recruiting	OC	Poly-ICLC +Neoantigen peptide (NeoVax)	Anti-PD-1(nivolumab)	Injection underneath the skin	Not specified; priming and booster
NCT02897765	I	Completed	UBC, BT, T CCB, MM, M, SC, NSCL	Neoantigen peptides (NEO-PV-01)	Anti-PD-1(nivolumab)	Subcutaneous	Not specified
NCT03289962	I	Recruiting	Solid Cancers	Neoantigen mRNA (RO7198457)	Anti-PD-L1(atezolizumab)	Intravenous infusion RNA-lipoplex,	25–100 mg qw priming and boosters
NCT03568058	I	Active, not recruiting	Advanced Cancer	Neoantigen peptide	Anti-PD-1(pembrolizumab)	None	None
NCT04266730	I	Not yet recruiting	NSCLC, SCCHN	Neoantigen peptide vaccine (PANDA-VAC) + Poly-ICLC	Anti-PD-1(pembrolizumab)	Subcutaneous	1,800 ug, 2,400 ug, priming and booster
NCT04161755	I	Recruiting	PC	Neoantigen mRNA (RO7198457)	Anti-PD-L1(atezolizumab)	None	None
NCT04072900	I	Recruiting	M (Skin)	Neoantigen peptide	Anti-PD-1(toripalimab)	None	4 × 3 mg all the peptides given by seven times
NCT03597282	I	terminated	Metastatic M	Poly-ICLC + neoantigen peptides (NEO-PV-01)	Anti-PD-1(nivolumab),	Subcutaneous	None
NCT02287428	I	Recruiting	GB	Neoanitgen peptide (NeoVax)	Anti-PD-1(pembrolizumab)	None	Not specific, priming and boost phases.
NCT04799431	I	Not yet recruiting	PC,MCRC	Neoantigen Vaccine + Poly-ICLC	Anti-PD-1(retifanlimab)	Subcutaneous	0.3 mg per peptide vaccine
NCT04248569	I	Recruiting	FLC	Neoanitgen peptide (DNAJB1-PRKACA fusion kinase)	Anti-PD-1(nivolumab)and anti-CTLA-4(ipilimumab)	None	Not specifically, Priming and booster
NCT03219450	I	Not yet recruiting	LL	Neoanitgen peptide (NeoVax)+ Poly-ICLC	Anti-PD-1(pembrolizumab)	None	Not specifically, priming and booster

(Continued)

TABLE 2 | Continued

ClinicalTrial.gov identifier	Phase	Enrollment status	Cancer Type	Neoantigen Formulation	Additional intervention	Delivery	Dose and Schedule
NCT03121677	I	Recruiting	FL	Personalized tumor vaccine+ Poly-ICLC	Anti-PD-1(Nivolumab)	Subcutaneous	Not specifically
NCT03166254	I	Withdrawn	NSCLC	Neoantigen peptides (NEO-PV-01)+ Poly-ICLC	Anti-PD-1(pembrolizumab)	None	Not specifically, priming and booster
NCT03199040	I	Recruiting	TNBC	Neoantigen DNA	Anti-PD-L1 (durvalumab)	None	None
NCT03532217	I	Active, not recruiting	MHSPC	Neoantigen DNA	Anti-PD-1(nivolumab)and anti-CTLA-4(ipilimumab)	Intramuscular	4 mg, priming and booster
NCT03380871	I b	Completed	Advanced or metastatic NSCLC	Neoantigen peptides (NEO-PV-01)+ Poly-ICLC	Anti-PD-1(pembrolizumab)	Subcutaneous	None
NCT02950766	I	Recruiting	High-risk RCC	Neoanitgen peptide (NeoVax)+ Poly-ICLC	Anti-CTLA-4(ipilimumab)	Subcutaneous	Not specifically, priming and booster
NCT03359239	I	Recruiting	UC/BC	Neoanitgen peptide (PGV001) + Poly-ICLC	Anti-PD-L1(atezolizumab)	None	Up to ten synthetic peptides—100 μg (0.01 ml, 10 mg/ml) per peptide. One tetanus helper peptide—100 μg (0.01 ml, 10 mg/ml),up to ten total doses
NCT03422094	I	Terminated	GBM	Neoanitgen peptide (NeoVax)+ Poly-ICLC	Anti-PD-1(nivolumab)and anti-CTLA-4(ipilimumab)	Subcutaneous	Not specifically, priming and booster
NCT03929029	Ib	Recruiting	M	Neoanitgen peptide (NeoVax)+ Poly-ICLC + Montanide	Anti-PD-1(nivolumab)and anti-CTLA-4(ipilimumab)	None	None
NCT04117087	I	Recruiting	CRC, PC	KRAS peptide+ Poly-ICLC	Anti-PD-1(nivolumab)and anti-CTLA-4(ipilimumab)	None	1.8 mg, priming and booster

ES-SCLC, extensive stage small cell lung cancer; NSCLC, non-small cell lung cancer; MCRC, metastatic colorectal cancer; CRC, colorectal cancer; MSSCR, microsatellite state colorectal cancer; UBC, urinary bladder Cancer; UC/BC, urinary/bladder carcinoma; BT, bladder tumors; TCCB, transitional cell carcinoma of the bladder; MM, malignant melanoma; M, melanoma; SC, skin cancer; SCCHN, squamous cell carcinoma of head and neck; HCC, hepatic cell cancer; TNBC, triple negative breast neoplasms; RCC, renal cell carcinoma; LL, lymphocytic leukemia; FL, follicular lymphoma; PC, pancreatic carcinoma; MHSPC, metastatic hormone-sensitive prostate cancer; FLC, fibrolamellar hepatocellular carcinoma; OC, ovarian cancer; GBM, glioblastoma; Poly-ICLC, polyinosinic–polycytidylic acid-poly-l-lysine carboxymethylcellulose.

adverse events also elevate. The key point is to find the balance between efficacy and safety (82–87). Owing to lack of more clinical data, further studies about dose and administrating route are urgently required (64). When it comes to the combination, a vital problem emerges, the sequence of administration of neoantigen vaccines and ICIs. Pre-vaccination promote baseline immunity, significantly increase expression of INF-γ, Fms-related tyrosine kinase 3 ligand (FLT3L) and granulocyte–macrophage colony-stimulating factor(GM-CSF), and help "cold" tumor, such as glioblastoma and pancreatic ductal carcinoma, respond to ICIs (65, 66, 71). Additionally, administration of ICIs at vaccination or post-vaccination can boost vaccine-induced immune response. Unfortunately, limited data is presented, so further endeavors are necessary to explore the proper sequence of administration. To monitoring antitumor efficacy, applications of gene sequencing and single-cell sequencing technologies might find why resistance appear and discover the alternative neoantigens.

CHALLENGES AND IMPROVEMENTS

Though the combination of neoantigen vaccines and ICIs is promising in personalized treatment, there are still many problems.

Selection of Population Who May Potentially Benefit From the Combination

ICIs and neoantigen vaccines alone can only benefit a fraction of patients, efforts to find out the most proper population to receive the combination therapy are urgently required (88).

Recent studies have shown that somatic mutation, neoantigen burden and neoantigen density associated with long-term benefit from immune checkpoint inhibitors in several solid tumors, which indicated that more mutation-associated neoantigens may enhance the immunogenicity and improve immune responses of ICIs (19, 44, 89). The possible explanation is that higher mutational burden provides a base to generate more immunogenic neoantigens. Furthermore, researchers have found there were neoantigen-specific T cells in the peripheral blood in patients with tumor regression, and this also demonstrated that some neoantigens indeed could activate T cell which had an antitumor effect (19, 44, 88, 90). Therefore, neoantigen burden may be a significant predictor for combinational immunotherapy to distinguishing responders from non-responders.

However, some studies also revealed that responders to ICIs were not restricted to patients with high neoantigen burden, which demonstrated that not only the quantity of neoantigens is significant, rather their "quality" is vital as well (91, 92). For example, in melanoma, an effective antitumor CD8+ T cells response can be produced by a few epitopes, which have affinity

to TCRs (26, 43). Another instance, renal cell carcinoma (RCC) has only a moderate to low mutation rate; however, it is universally known that RCC has a good response to immunotherapy (93). Researchers tried to explain this phenomenon and discovered that RCC possess the highest level of insertion and deletion type (indel) mutations, which are regarded to frequently create a new open reading frame and a higher proportion of neoantigens (94). Considering the quality of mutation into the prediction is also significant.

What's more, lower neoantigen intra-tumor heterogeneity (ITH) is correlated with significantly longer progression-free survival. Neoantigen heterogeneity has a high variable rate with an average of 44% neoantigens found heterogeneously, in a subset of tumor regions (range of 10 to 78%) (55). Researchers have observed that lower hazard ratios when considering both neoantigen burden and ITH compared with the use of neoantigen burden alone to screen the potential population (55). Additionally, tumors with low ITH had an elevated PD-L1 expression (55). Thus, combining heterogeneity selecting a proper threshold with neoantigen burden is critical to find out the population who may benefit from ICIs and neoantigen vaccines.

Effectively controlling tumors with personalized vaccines and ICIs, is associated with neoantigen-specific T cells, which can exist in peripheral blood and show a phenotype different from patients without a response. Further studies to investigate characterization of neoantigen-specific T cells in responder patients, showed that there was relatively high expression of the activation markers CD161, TIGIT, 2B4 and KLRG1,low level of expression of inhibition markers CD27, CD28 and CD127, and a high level of co-inhibitory molecules, including PD-1 (95, 96). These results indicate that identification and characterization of neoantigen-specific T cells may contribute to predict who will response to the combination therapy.

Neoantigens Heterogeneity and Dynamic Variation of Neoantigen Landscape

Neoantigens arise from tumor-specific mutations, and they are variable in different tumors or patients. ITH has a significant impact on the response to immunotherapies. In addition, in patients who initially respond to ICIs, tumors could experience an evolution of epitopes, which will alter the neoantigen landscape and lead to sequential resistance. More importantly, mechanisms of ITH and the dynamic variation of neoantigens landscape are unclear for now (13, 97, 98). We just observed that high heterogeneity may lead to a poorer response to both vaccination and ICIs. For this problem, researchers provide that designing a neoantigen vaccine with multiple targets and based on the variation landscape may help overcome ITH and the dynamic alteration (55).

Identification of Potential Neoantigens

Exact identification of the neoantigens is capable of producing potent epitopes which can be recognized specifically by TCRs and induce strong immune response. However, this is difficult to achieve in current silico predicting systems (49). Some studies showed that in predicted neoantigens, only a fraction (20%) had immunogenicity, demonstrating the low accuracy of current neoantigens prediction algorithms (99, 100). For now, there is no single method providing an accurate and reliable prediction and identification of neoantigens. The possible solutions for this problem are as following. One method is designing multi-epitope vaccines. Using of a platform which can accommodate a large number of neoantigens, may help overcome limits of the prediction algorithms. In a study, combing treatment with the adenoviral vaccine targeting 31 neoantigens increased the number of mice with complete regression (~50%), which may be due to the increasing specific T-cell clones (8). These findings demonstrate that multi-epitope vaccines may be a solution for inaccuracy of the bioinformatics tools for predicting neoantigens (8). Another option is to broaden our analysis of potential neoantigens to include other types of potentially immunogenic alterations, for current predicted neoantigens are mainly resulting from missense mutations (2). For example, chromosomal insertions, inversions, and translocations can lead to fusion transcripts, which exist in certain cancers, such as chronic myelogenous leukemia, lung cancer, bladder cancer, and ovarian cancer (101, 102).

Faster and Cost-Effective Vaccine Production

Currently, although the emergence of NGS, WES and other techniques improve the identification and production of neoantigen vaccines, verifying neoantigens and vaccines generation are still time-consuming and expensive, and usually 3–5 months are required to prepare vaccines from tumor samples (26, 43). Additionally, problems that the identification of neoantigens needs lots of tumor tissues, while the yield of usable epitopes or neoantigens is very low, and it is difficult to solve for technical limits (103). These obstacles have been largely hampering the development of neoantigen vaccines in clinical settings. There is an urgent need to develop better neoantigen prediction algorithms and manufacturing technologies which can decrease the price and shorten the time (4, 104). Efforts for this work are going on, and Anna have established a fast process assembling 60 unique patient mutanome-specific neoantigens and producing personalized adenoviral vaccines within 6 weeks from the time of patient biopsy (16).

Safe and Efficient Delivery System

To generate a potent neoantigen vaccine, a safe and efficient delivery system is needed, which can help induce strong immune response. Effector T cells can be induced by the specific antigens or epitopes within the tumor cells and neoantigen-specific T cells clonal expansion in tumors symbols the effective antitumor response. Neoantigen vaccines based on virus could be the proper candidates to produce potent antitumor immunity. Many studies have proven adenoviruses were powerful genetic vaccine platforms with unique feature encoding for large antigen to activate effective CD8+ and CD4+ T-cell responses safely (105–107). In a preclinical study, a adenoviral vaccine encoding 31 neoantigens, the largest number used so far for neoantigen-based vaccines, selected from the murine CT26 colon carcinoma cell line, produced potent antitumor immune responses, and more than 1,000 antigen-specific IFN-γ

secreting lymphocytes/million splenocytes, CD8+ and CD4+ T lymphocytes were generated (16).

Low Efficacy of Neoantigen Vaccines

In preclinical trials, researchers did not detect neoantigen-specific T cells in untreated mice with tumors. The possible reasons are showed below: the inadequate mutated gene expression; antigen can't be presented effectively due to low affinity to HLA or TCRs on T cells; dynamic variation of neoantigen landscape leading to dominant neoantigens eliminated; T cells in the repertoire which can bind to mutant neoepitopes are in short or undergoing apoptosis (65). Two approaches are capable of enhancing neoantigen-specific T cell responses and improving the antitumor potency.

One is to use a potent adjuvant to stimulate innate immunity. In some preclinical studies, researchers found that adding an agonist OX40 antibody to PancVAX, decreased T cell exhausted markers, such as Lag3 and PD-1, and helpedCD4+ T cell avoid immunosuppressive Treg phenotype (108, 109). What's more, FoxP3+CD4+T cells decreased and tumor-specific IFN-γ-secreting CD4+ T cells appeared when combining PancVAX with OX40 (65). Furthermore, OX40 is capable of increasing the survival rate of antitumor T cells with low avidity (94). These findings suggest that an effective adjuvant can produce neoantigen-specific TILs, help activate T cells and maintain TILs through survival improvement.

Another strategy to improve the efficacy of neoantigen vaccines is taking MHC class II peptides into vaccination design (88). The clonal expansion of tumor-specific T cells with potent antitumor ability is the core of the success in the personalized cancer immunotherapy (49). Kreiter and colleagues conducted a study suggesting that many personalized cancer vaccines with immunogenic neoantigens were correlated to MHC class II molecules on CD4+ helper T cells (110). In the study, PancVAX played its role to control or shrink tumors primarily through CD8+ T cells, meanwhile, CD4+ T cells may have influence as well. Researchers further investigated the influence of both CD8+ T cells and CD4+ T cells, and noted that antitumor response disappeared when depleting CD8+ T cells while partial responses loss with CD4+ T cells eliminated. The successful example of immunotherapy in melanoma was due to existence of epitopes targeting both CD4+ and CD8+ T cells (16, 20, 26, 65). Therefore, vaccination with neoantigens targeting both CD8+ T cells and CD4+ T cells is vital to induce powerful antitumor immune responses, for the primary immune driver CD8+ T cells can work better with the synergy of CD4+ T cells (111, 112).

Dose and Sequence of ICIs and Vaccines

Though many preclinical and clinical trials investigate the safety and efficacy of the combination of ICIs and neoantigen vaccines, dose and schedule for ICIs and vaccines have been minimally studied. Some researchers found that after the response to interferon secreted by T cells, both PD-1 on activated T cells and PD-L1 on tumors emerged in a short time, which supports that using ICIs first or concomitant with vaccines may be both rational (113). In some studies, after vaccination with neoantigens in patients, the expression of both PD-1 on neoantigen-specific T cells and PD-L1 in tumor tissues increased, and anti-PD-1 or anti-PD-L1 immunotherapy improved the efficacy of vaccines, suggesting that administering neoantigen vaccines before ICIs may have a greater opportunity to achieve the maximal antitumor response (1, 13, 20, 36, 53–55). We have searched up many preclinical and clinical trials, unfortunately, we didn't find a standard sequence or dose for the combination, which need further exploration.

CONCLUSION

Neoantigen vaccines alone have a limited efficacy, and ICIs has been limited to a minority of patients with certain cancer types. However, the combination of personalized vaccines and ICIs can significantly improve the antitumor efficacy with minimal additional toxicity compared to either single method by improving sensitivity and efficacy of ICIs, overcoming acquired resistance of ICIs and relieving suppressive microenvironment. This has led to envisaging and developing combined strategies that might augment tumor regression and prolong overall survival for patients with metastatic cancer. However, there still some important aspects for the combination to achieve the maximal efficacy, including optimizing the identification, predication and production of neoantigen vaccines, selecting proper population for the combination therapy, and the optimized dose and sequence of the two agents.

AUTHOR CONTRIBUTIONS

SZ contributed conception and overall idea of the study. J-YL wrote the first draft of the manuscript. SZ wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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ERO1L Is a Novel and Potential Biomarker in Lung Adenocarcinoma and Shapes the Immune-Suppressive Tumor Microenvironment

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Background: The endoplasmic reticulum oxidoreductin-1-like (*ERO1L*) gene encodes an endoplasmic reticulum luminal localized glycoprotein known to associated with hypoxia, however, the role of *ERO1L* in shaping the tumor immune microenvironment (TIME) is yet to be elucidated in lung adenocarcinoma (LUAD).

Methods: In this study, raw datasets (including RNA-seq, methylation, sgRNA-seq, phenotype, and survival data) were obtained from public databases. This data was analyzed and used to explore the biological landscape of *ERO1L* in immune infiltration. Expression data was used to characterize samples. Using gene signatures and cell quantification, stromal and immune infiltration was determined. These findings were used to predict sensitivity to immunotherapy.

Results: This study found that *ERO1L* was significantly overexpressed in LUAD in comparison to normal tissue. This overexpression was found to be a result of hypomethylation of the *ERO1L* promoter. Overexpression of *ERO1L* resulted in an immune-suppressive TIME *via* the recruitment of immune-suppressive cells including regulatory T cells (T_{regs}), cancer associated fibroblasts, M2-type macrophages, and myeloid-derived suppressor cells. Using the Tumor Immune Dysfunction and Exclusion (TIDE) framework, it was identified that patients in the $ERO1L^{high}$ group possessed a significantly lower response rate to immunotherapy in comparison to the $ERO1L^{low}$ group. Mechanistic analysis revealed that overexpression of *ERO1L* was associated with the upregulation of JAK-STAT and NF-κB signaling pathways, thus affecting chemokine and cytokine patterns in the TIME.

Conclusions: This study found that overexpression of *ERO1L* was associated with poor prognoses in patients with LUAD. Overexpression of *ERO1L* was indicative of a hypoxia-induced immune-suppressive TIME, which was shown to confer resistance to

immunotherapy in patients with LUAD. Further studies are required to assess the potential role of *ERO1L* as a biomarker for immunotherapy efficacy in LUAD.

Keywords: *ERO1L*, tumor microenvironment, biomarker, immunotherapy, lung adenocarcinoma

INTRODUCTION

Lung cancer is a leading cause of cancer-related mortality and lung adenocarcinoma (LUAD) accounts for approximately 50% of all reported cases (1). In recent years, as precision medicine is becoming a reality, LUAD treatments have gradually evolved from empirical chemotherapy to personalized therapies. Immunotherapies, which have the advantage of high efficiency, long duration, and low toxicity, have led to a paradigm shift in cancer treatment. Immunotherapy has become the standard of care for advanced LUAD. However, widespread usage of immunotherapy is limited and drug resistance is increasingly reported (2, 3). As a result, the identification of biomarkers to enable patient selection is urgently required.

There is a growing body of evidence supporting the theory that the tumor immune microenvironment (TIME) plays a crucial role in the response to immunotherapy. The TIME comprises a series of infiltrating cells, such as neoplastic cells, immune cells, endothelial cells, fibroblasts. Different infiltration components are associated with different clinical outcomes. Based on immune score, recent research has classified TIME into three subtypes: immune-inflamed (I-I TIME), immune-desert (I-D TIME), and immune-excluded (I-E TIME) (4). Patients with I-I TIME are frequently reported to be infiltrated with an abundance of inflammatory cells. This indicates that they will have a significant clinical response to immune checkpoint inhibitor (ICI) therapy. Contrastingly, I-D TIME and I-E TIME are both be considered noninflamed TIME. As such, these patients are rarely responsive to ICI therapy (5). The differential responses of these subtypes present the need to develop individualized treatment strategies. However, two key challenges remain. Firstly, determination of the threshold for an inflamed or noninflamed TIME. And secondly, the lack of appropriate biomarkers that are able to distinguish TIME subtypes (6).

The endoplasmic reticulum oxidoreductin-1-like (*ERO1L*) gene, which is located on chromosome 14 in humans, is considered to be the primary source of the endoplasmic reticulum. ERO1L is an endoplasmic reticulum luminal localized glycoprotein which favors disulfide bond formation *via* the selective oxidization from protein disulfide isomerases (7). Hypoxia is a hallmark of the tumor microenvironment and is reported in the majority of tumors overexpressing *ERO1L* (8). Hypoxic stress has been described to cause immunosuppression by controlling angiogenesis. This is predicted to result in resistance to ICI therapy (9). What's more, *ERO1L* is known to promote programmed death-ligand 1 (PD-L1) expression by increasing the expression of hypoxia-inducible factor1α (HIF-1α) and subsequently facilitating oxidative protein folding within PD-L1. Ultimately, this results in immune escape (10). The role of *ERO1L* in the crafting of the tumor immunological microenvironment is yet to be elucidated.

In this study, the association between *ERO1L* expression and TIME was investigated in LUAD. Bioinformatics techniques including cell quantification algorithms and gene expression profiling were used. This study identified that the overexpression of *ERO1L* is a feature in an immune-suppressive TIME. This provided insight into the potential association between *ERO1L* and tumor-immune interactions.

MATERIALS AND METHODS

mRNA and Protein Expression Analyses of *ERO1L* Using Public Databases

The *ERO1L* mRNA expression in pan-cancer was analyzed in the Oncomine database (www.oncomine.org) with the following thresholds: *p*-value of 0.001, a fold change of 1.5, and a top 10% gene ranking (11). The expression data belonging to four datasets (GSE7670, GSE31210, GSE32863, and GSE19188) were downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). Expression profiles were normalized by z-scores of $\log_2$(count+1). Using the *limma* package in R-4.0.3, batch normalization was also completed. Protein expression levels were analyzed using the HPA database (http://www.proteinatlas.org). Antibodies used in the HPA database included HPA026653 (Sigma-Aldrich), HPA030053 (Sigma-Aldrich) and CAB034294 (Santa Cruz Biotechnology). These three antibodies were all validated by orthogonal method (antibody staining mainly consistent with RNA expression data across 41 tissues) and by independent antibodies (protein distribution across 45 tissues similar between the independent antibodies HPA026653 and HPA030053).

TCGA Database Analysis

TCGA LUAD data was downloaded from the UCSC Xena database (http://xena.ucsc.edu/). This data included information on: RNA-seq (HTSeq-FPKM), DNA methylation (Illumina Human Methylation 450), and clinical profiles (including both phenotype and survival data). Expression levels were normalized using the z-score of $\log_2$(FPKM+1) to exclude potential bias. Patients were assigned into *ERO1L*-high and *ERO1L*-low groups based on the median expression value according to the RNA sequencing data. In terms of methylation analysis, we included 18 methylation sites (**Table S4**). These sites were mapped to the *ERO1L* gene using the UCSC Genome Browser HG19 RefSeq database (http://genome.ucsc.edu). The methylation level of each CpG site was recorded as a β value. This value indicated the ratio of the methylated signal intensity over the sum of the methylated and unmethylated

intensities at each locus. Each locus with an average β-value of less than 0.20 was considered a hypomethylation site. In addition, Kaplan-Meier survival curves were plotted to show differences in survival time. For this data, log-rank *p*-values reported in the *survival* package in R-4.0.3 were used to determine statistical significance. Path analysis was performed in the *ggalluvial* in R-4.0.3. Visualization was performed using the OriginPro 2019b software (version 9.6.5.169).

Organoid Culture

Mouse lung tissue was carefully dissected avoiding other tissue contamination, then minced with surgical scalpels and incubated in Trypsin (Gibco) at 37°C for 40-50 min. The digestion was terminated with 10% serum (Gibco), and digested tissues were filtered with a 70 μm cell strainer in order to filter out debris that had not been fully digested. Cells were resuspended with Matrigel (Corning) and plated in 48 well plates. Then the Matrigel was solidified in an incubator at 37°C for 15-20 min and overlaid with 150-200 μL medium. Organoid medium contains advanced DMEM/F12, FGF 100 ng/mL, EGF 10 ng/mL, B27 supplement (2 X final), N2 supplement (1 X final), Noggin 100 ng/mL, RSPO-1 (10% final), Wnt-3a (10% final), Y27632 10 μM,A83-01 10 μM, Glutamax (1 X final) and Penstrep (1 X final). Cultures were kept at 37°C, 5% CO2 in an incubator and the medium was exchanged every 4-8 days according to the number of spheres. For passaging, the Matrigel containing organoids was dissolved in 3-5 mL TrypLE at 37°C for 10 min, and pipetted vigorously (80-100 times) to dissociate organoids into single cells. Cells were filtered with 70 μm cell strainer, centrifuged at 1,500 rpm for 10 min and resuspended with Matrigel.

Organoid Infection

Organoids were dissociated into single cells as described above, resuspended with 200 μL medium ae well as 2-3 mL virus particles, and added polybrene to 1 X final. After being centrifuged at 2,000 rpm, 37°C for 1 hour, the cells were then incubated at 4°C, 5% CO_2 for 2-3 hours. Finally, an organoid culture was performed as described above.

Co-Expression Module Identification and Pathway Analysis

Firstly, genes which were co-expressed were identified in the Oncomine and TCGA databases respectively. By overlapping the results from these two databases, we identified a co-expression module consisting of 17-genes. STRING (version 10.5) was used to construct a protein-protein interaction (PPI) network. The 17 genes within the module were subjected to pathway enrichment analysis using DAVID (https://david-d.ncifcrf.gov/) (12). Results were visualized using the *Hmisc and ggplot2* packages in R-4.0.3. Gene set enrichment analysis (GSEA) was performed using the GSEA software (version 4.1.0) and Broad's GSEA algorithm (13).

Immune Infiltration Analysis

The relationship between *ERO1L* expression levels and immune infiltration was initially determined using the TIMER2.0 database (http://timer.comp-genomics.org). The TIMER2.0 database utilizes *immunedeconv*, which is an R package integrating six state-of-the-art algorithms. These algorithms include: TIMER, xCell, MCP-counter, CIBERSORT, EPIC, and quanTIseq (14). These algorithms were systematically benchmarked, and each was found to have unique properties and strengths. *ERO1L* expression was analyzed in the presence of seven types of immune infiltrating cells, including B cells, $CD4^+$ T cells, $CD8^+$ T cells, NK cells, macrophages, CAFs, and MDSCs. During immune infiltration analysis, adjustments were also made for tumor purity. The online tool CIBERSORTx (https://cibersortx.stanford.edu) was used to estimate different immune cell proportions (15).

Correlation analysis between the expression of *ERO1L*, immune cell markers and cytokines as well as chemokines was also performed using the TIMER2.0 database, specifically using the Gene_Corr module. The functionality of this module allows users to uncover the co-expression pattern of genes across TCGA cancer types. When provided with one initial gene of interest and up to 20 other genes, the TIMER2.0 database generates a heatmap table of Spearman's correlation of gene expression between the gene of interest and the other input genes. After adjustment for tumor purity, a Spearman's $\rho > 0$ with *p*-value <0.05 was considered as a positive correlation and a Spearman's $\rho < 0$ with *p*-value <0.05 was considered as a positive correlation. Secondary confirmation of correlation analysis was performed using GEPIA (http://gepia.cancer-pku.cn) (16). Tertiary confirmation of correlation analysis was performed using TCGA expression data from the GEPIA database (http://gepia.cancer-pku.cn/index.html).

Single-Cell Sequencing Analysis

Processed gene expression data was download from the GEO database (GSE99254). This project consists of deep single-cell transcriptome data with complete T cell receptor information, which identified multi-dimensional characteristics of infiltrating lymphocytes (17). Single-cell transcriptome data was analyzed based on t-SNE dimension reduction using the R package *Rtsne*. Additionally, the Tumor Immune Single-cell Hub (TISCH) database was used to analyze the correlations between *ERO1L* expression and infiltrating immune cells (18). TISCH is a scRNA-seq database focusing on the tumor microenvironment. This database includes 79 datasets and 2,045,746 cells. TISCH provides detailed cell-type annotation at the single-cell level, enabling detailed exploration of the tumor microenvironment across various different cancer types.

Immunotherapy Response Prediction

In the first instance, in order to estimate the presence of the various immune cell populations in the LUAD tissues, the R package *ESTIMATE* was used. Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data (ESTIMATE) is a tool that predicts tumor purity *via* the use of gene signatures. The tool calculates three scores, including stromal score, which predicts the presence of stromal cells in tumor bulk; immune score, which infers the levels of immune cells infiltration in tumor tissue; and estimate score, which estimates tumor purity. Subsequently, the R package

MCPcounter was used to develop a more detailed idea of the level of immune cell infiltration. Microenvironment Cell Populations-counter (MCPcounter) is a quantification method that determines the relative abundance of an immune cell in heterogeneous tissues. This method uses marker genes optimized for interrogating microarray data (19). In order to predict the response to immune checkpoint blockade, Tumor Immune Dysfunction and Exclusion (TIDE) score was employed. TIDE is a computational method used to model two primary mechanisms of tumor immune evasion. These mechanisms are: the induction of T cell dysfunction in tumors with high infiltration of cytotoxic T lymphocytes (CTL) and the prevention of T cell infiltration in tumors with low CTL level (20, 21). Using this framework and RNA-Seq tumor expression profiles, TIDE can predict the outcomes of non-small cell lung cancer (NSCLC) patients treated with first-line anti-PD1 or anti-CTLA4 more accurately than other biomarkers such as PD-L1 levels and mutational load.

Statistical Analysis

Chi-squared and Fisher's exact tests were used to investigate the significance of the correlation of *ERO1L* expression with clinicopathological features in LUAD patients. Analysis was performed using SPSS (version 23.0). ANOVA was used to identify the *ERO1L* expression levels in different datasets (22). The correlation of gene expression was evaluated using Spearman's correlation coefficient (23). A p value <0.05 was considered statistically significant.

RESULTS

Quantification of *ERO1L* mRNA Expression in Pan-Cancer

In order to determine the mRNA expression profile of *ERO1L* in pan-cancer, expression levels of *ERO1L* in the Oncomine database were analyzed. Comparisons of mRNA expression levels of *ERO1L* in pan-cancers versus normal tissue identified nine types of cancer in which *ERO1L* mRNA expression levels were elevated. These types of cancer included bladder, brain, central nervous system, colorectal, gastric, kidney, lung, lymphoma, ovarian, and pancreatic cancer. In addition, three types of cancer in which *ERO1L* mRNA expression levels were diminished were identified. These types of cancer included esophageal cancer, head and neck cancer, and leukemia (**Figure 1A**). What's more, lung cancer was found to be associated with a significantly higher expression level of *ERO1L* in comparison to normal tissue. The expression levels of *ERO1L* were increased in seven datasets while no dataset possessed decreased levels of *ERO1L*.

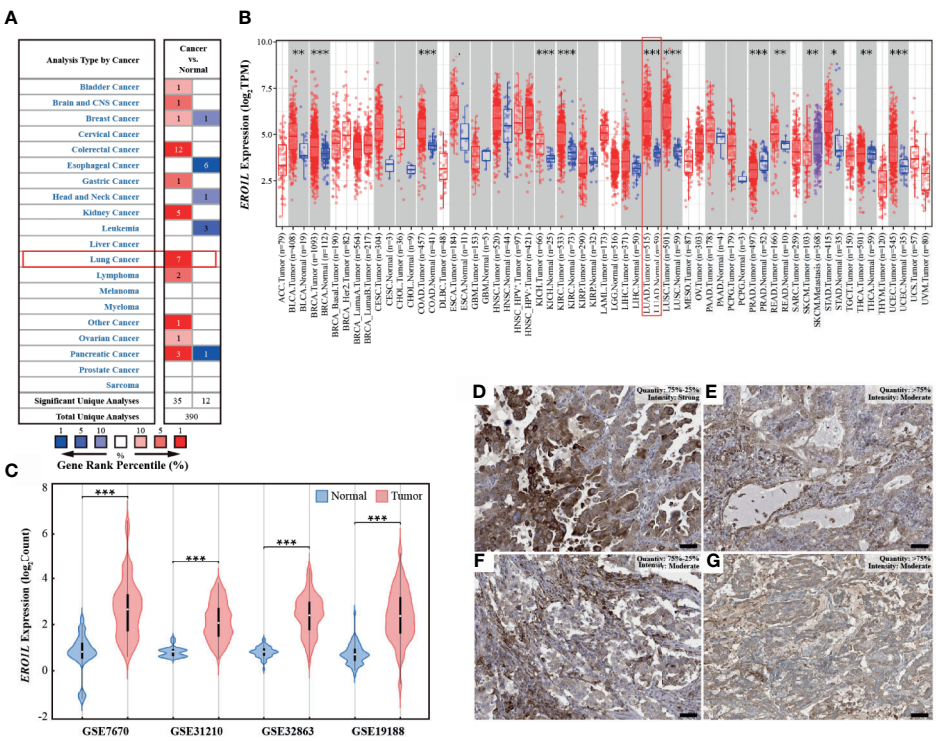

FIGURE 1 | *ERO1L* expression levels in pan-cancer and LUAD. **(A)** Expression levels of *ERO1L* mRNA in pan-cancer, compared with normal tissues in the Oncomine database. The number in each cell denotes the number of datasets. **(B)** Expression levels of *ERO1L* mRNA in pan-cancer, compared with normal tissue from the TCGA database. **(C)** Expression levels of *ERO1L* mRNA in LUAD across four GEO datasets. **(D-G)** Expression levels of *ERO1L* protein in four patients with LUAD from the HPA database. Scale bar: 50 μm. *p < 0.05; **p < 0.01; ***p < 0.001.

To further investigate the mRNA expression levels of *ERO1L* in pan-cancers, RNA-sequencing data from The Cancer Genome Atlas (TCGA) program was analyzed. Interestingly, it was discovered that *ERO1L* mRNA expression levels were somewhat increased in pan-cancers in comparison to normal tissues (**Figure 1B**). This finding is consistent with our analysis of data from the Oncomine database, which revealed significantly elevated expression levels of *ERO1L* in lung adenocarcinoma (LUAD; p <0.001) and in lung squamous cell carcinoma (LUSC; p <0.001).

Expression Profiles of *ERO1L* mRNA and Protein in LUAD

In order to study the mRNA expression levels of *ERO1L* in LUAD, further analysis was performed on datasets from the Gene Expression Omnibus (GEO) database. In this analysis, four datasets (GSE7670, GSE31210, GSE32863, and GSE19188) comprising a total of 526 samples were included. This included 356 tumoral and 170 paired normal biopsies (**Table S1**). After normalizing the expression profile, it was identified that *ERO1L* mRNA expression levels in LUAD was significantly elevated in comparison to normal tissues. This was observed in all of the datasets analyzed. Interestingly, expression fold changes ranged from 2.8 to 4.1 times (**Figures 1C** and **S1A**). In addition, the mRNA expression of *ERO1L* in LUAD was investigated using the TCGA program. Similarly, significantly elevated expression levels of *ERO1L* mRNA were observed in LUAD in comparison to normal tissues. This was observed when the analysis was performed using both TCGA program and the Genotype-Tissue Expression (GTEx) program (**Figure S1B**).

These elevated *ERO1L* mRNA expression levels were confirmed in LUAD. As a logical next step, the protein expressions of *ERO1L* in LUAD were then investigated. Analysis using The Human Protein Atlas (HPA) program revealed that ERO1L was positively detected *via* immunohistochemistry (IHC) staining in patients with LUAD. Eleven patients with LUAD were identified in the HPA database, all of these patients possessed positive ERO1L protein expression (**Figures 1D–G**). Out of these patients, the intensity of IHC staining is as follows: three patients were associated with strong intensity, six with moderate, and two with weak intensities of IHC staining (**Table S2**).

Overexpression of *ERO1L* Is Associated With a Poorer Prognosis in LUAD

In order to study the correlation between *ERO1L* expression and prognosis in LUAD patients, six cohorts of patients were obtained from the PrognoScan database (**Table S3**). Via the analysis of hazard ratios (HR) and 95% confidence intervals (CI), four cohorts of LUAD patients (HLM, Nagoya, UM, and NCCRI) with high expression of *ERO1L* were identified. This high expression of *ERO1L* was associated with worsened prognoses in these patients as measured by overall survival (OS) and recurrent-free survival (RFS). Similarly, analysis was performed on survival data from the Kaplan-Meier plotter database. This is based on the Affymetrix microarrays with probe ID 218498_s_at for the *ERO1L* gene. These results showed consistently that overexpression of *ERO1L* was associated with worse prognoses in patients with LUAD in terms of OS (HR: 1.52, 95% CI: 1.27-1.82; **Figure 2A**) and RFS (HR: 1.93, 95% CI: 1.47-2.53; **Figure 2B**).

The following hypothesis was proposed: *ERO1L* is a potential biomarker in patients with LUAD. To investigate this hypothesis, survival analysis was applied to the RNA-sequencing data obtained from the TCGA program. This analysis revealed that there was a significant correlation between *ERO1L* overexpression, shorter

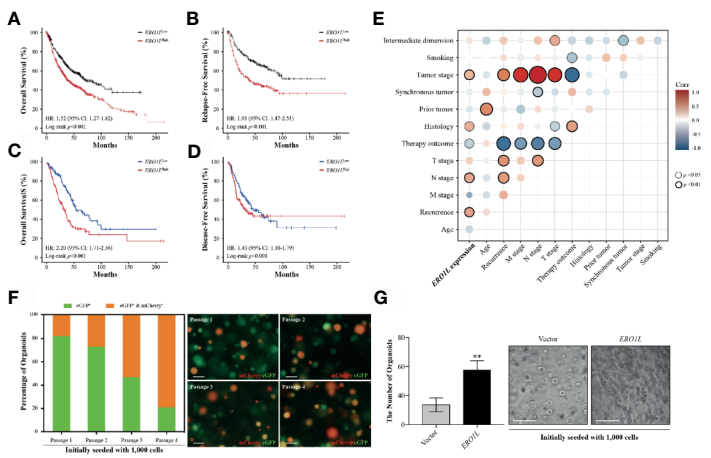

FIGURE 2 | Overexpression of *ERO1L* predicts a poorer prognosis in LUAD. **(A, B)** Survival analysis comparing low and high expression levels of *ERO1L* in the Kaplan-Meier plotter database. Overexpression of *ERO1L* was correlated with significantly poorer overall survival **(A)** and relapse-free survival **(B)**. Overexpression of *ERO1L* was correlated with significantly poorer overall survival **(C)** and disease-free survival **(D)**. **(E)** Association between the expression of *ERO1L* and clinicopathological characteristics in patients with LUAD. **(F)** Fluorescence images of *ERO1L*-overexpressed organoids show an increase in the number of chimeric organoids over passaging. **(G)** Quantification of *ERO1L* upregulated organoids relative to the average number of organoid spheres in four random fields. $^{**}p < 0.01$

overall survival (HR: 2.20, 95% CI: 1.71-2.56; **Figure 2C**) and disease-free survival (HR: 1.43, 95% CI: 1.10-1.79; **Figure 2D**). Interestingly, *via* correlation and multiple linear regression analysis, it was found that the expression level of *ERO1L* correlated with specific clinicopathological characteristics in LUAD patients (**Figure 2E**). As shown in **Table 1**, *ERO1L* overexpression was significantly correlated with tumor recurrence, pathologic N stage, primary treatment outcome, tumor histology, and tumor stage.

Furthermore, we applied an organoid model to study the biological function of *ERO1L*. We designed a protocol for organoid infection through dissociating organoid spheres into single cells and then co-culturing with virus particles. By introducing cDNA encoding *ERO1L* (labeled with mCherry) into organoids (labeled with eGFP), we obtained *ERO1L*-overexpressed organoids labeled in different colors. Organoids would turn red when transduced with cDNA, indicating the overexpression of *ERO1L* (**Figure 2F**). Using this, we detected infected organoids and calculated the ratios of chimeric organoids from passage 1 to 4. The initial percentage of chimeric organoids was about 16%, and it gradually increased to 75% after passaging three times (**Figure 2F**). Besides,

TABLE 1 | Correlation between ERO1L and clinicopathological characteristics in patients with lung adenocarcinoma.

Clinicopathological Characteristics	*ERO1L* Expression		*p*-value
	Low	High	
Age (year)	65.13 ± 10.10	63.86 ± 10.74	0.262
Smoking status			
Years smoked	31.65 ± 13.23	28.31 ± 12.71	0.168
Cigarettes/day	2.23 ± 1.51	2.19 ± 1.34	0.855
Tumor dimension			
Intermediate dimension	0.79 ± 0.33	0.80 ± 0.33	0.832
Longest dimension	1.24 ± 0.54	1.22 ± 0.61	0.791
Shortest dimension	0.39 ± 0.18	0.38 ± 0.15	0.593
Tumor recurrence	91 (28.4%)	40 (43.0%)	0.008
Tumor stage			0.026
Stage I	211 (59.2%)	49 (45.8%)	
Stage II	79 (22.1%)	32 (29.9%)	
Stage III	51 (14.3%)	22 (20.6%)	
Stage IV	16 (4.4%)	4 (3.7%)	
T stage			0.574
T1	131 (36.7%)	32 (29.9%)	
T2	183 (51.3%)	61 (57.0%)	
T3	31 (8.7%)	9 (8.4%)	
T4	12 (3.4%)	5 (4.7%)	
N stage			0.020
N0	248 (69.3%)	60 (55.6%)	
N1	66 (18.4%)	27 (25.0%)	
N2	44 (12.3%)	20 (18.5%)	
N3	0 (0.0%)	1 (0.9%)	
M stage			0.746
M0	227 (93.8%)	73 (94.8%)	
M1	15 (6.2%)	4 (5.2%)	
Primary treatment outcome			0.040
Progressive disease	44 (15.7%)	22 (28.6%)	
Stable disease	27 (9.6%)	4 (5.2%)	
Partial remission	4 (1.4%)	0 (0.0%)	
Complete remission	205 (73.2%)	51 (66.2%)	
Tumor histology			0.028
Adenocarcinoma	203 (56.5%)	76 (70.4%)	
Adenocarcinoma with mixed subtypes	80 (22.3%)	15 (13.9%)	
Acinar cell carcinoma	17 (4.7%)	3 (2.8%)	
Bronchiolo-alveolar carcinoma, non-mucinous	17 (4.7%)	1 (0.9%)	
Papillary adenocarcinoma	16 (4.5%)	4 (3.7%)	
Mucinous adenocarcinoma	15 (4.2%)	2 (1.9%)	
Bronchiolo-alveolar carcinoma, mucinous	4 (1.1%)	1 (0.9%)	
Micropapillary carcinoma	2 (0.6%)	0 (0.0%)	
Bronchiolo-alveolar adenocarcinoma	2 (0.6%)	1 (0.9%)	
Solid carcinoma	2 (0.6%)	4 (3.7%)	
Signet ring cell carcinoma	1 (0.3%)	0 (0.0%)	
Clear cell adenocarcinoma	0 (0.0%)	1 (0.9%)	
Prior malignancy	61 (17.0%)	15 (13.9%)	0.444
Synchronous malignancy	7 (2.2%)	2 (2.0%)	0.943

the doubling time of organoid cells decreased from five to six days at passage 1 to two to two and a half days at passage 4. Transduction of cDNA of *ERO1L* in organoids was also performed. One thousand cells in each group were seeded and cultured for fourteen days (passage 2). Sphere formation was enhanced from cells overexpressing *ERO1L* compared with the control (**Figure 2G**). Hence, we concluded that organoids with *ERO1L* overexpression gradually gained an advantage in development, which could be extended over time.

Regulation of *ERO1L* mRNA Level *via* Promoter Methylation

In order to elucidate the mechanism underlying *ERO1L* expression, promoter methylation levels of *ERO1L* were investigated in 503 samples. This was performed *via* analysis of methylation profiles (Illumina Human Methylation 450) from the TCGA program (**Table S4**). A significant decrease in the methylation level of the *ERO1L* was identified in the promoter region in LUAD tissues in comparison to normal tissues (**Figure 3A**). Tumor stage subgroup analysis revealed that levels of *ERO1L* promoter methylation were most significantly decreased in stage IV patients (**Figure 3B**). In addition, correlation analysis revealed a significant negative correlation between mRNA levels and methylation levels of *ERO1L* (Spearman's ρ: -0.25, p <0.001). This confirmed that *ERO1L* mRNA expression was regulated by promoter methylation in LUAD patients (**Figure S1C**).

In order to analyze the clinical outcomes associated with methylation and expression of *ERO1L*, datasets of 468 patients in the TCGA database were analyzed. These datasets all contained data corresponding to methylation, expression, and survival profiles. Path analysis *via* a Sankey diagram was performed. This quantified and visualized the transitions with various lines and widths, and described paths and patterns across tumor stages, promoter methylation levels, *ERO1L* mRNA expression, and survival status (**Figure 3C**). As a result, it was determined that hypomethylation of the *ERO1L* promoter potentially induced overexpression of *ERO1L* mRNA and finally led to poor prognoses in individuals with LUAD. This pattern was observed more significantly in patients with advanced stages of cancer (**Figure S2A**). This data is consistent with the survival analysis based on *ERO1L* expression. This provides compelling evidence that *ERO1L* is associated with poor prognoses in patients with LUAD.

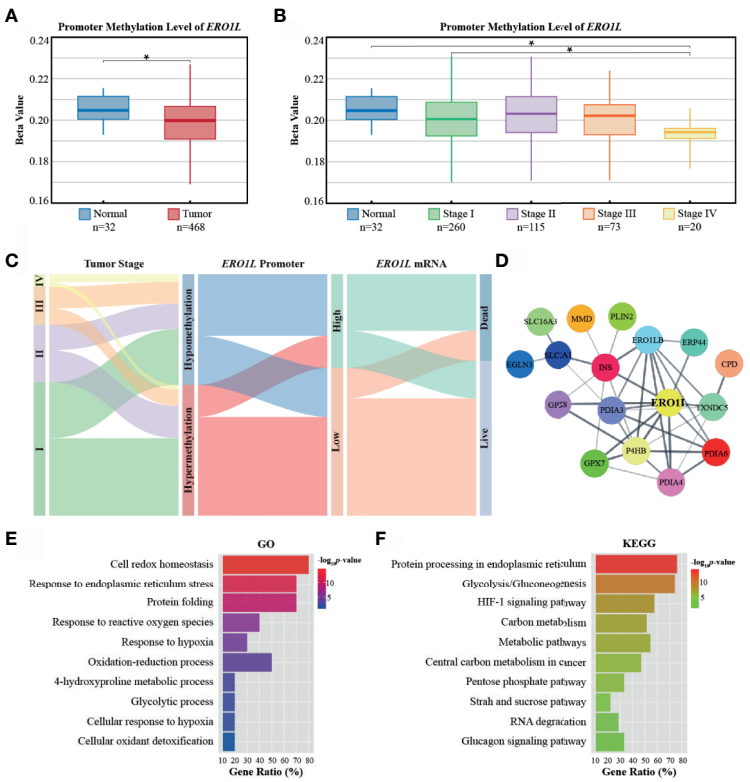

FIGURE 3 | Promoter methylation of *ERO1L* and functional annotation of the *ERO1L* co-expression module. **(A, B)** Analysis of *ERO1L* methylation in the TCGA database. Promoter methylation levels of *ERO1L* in tumor and normal tissues **(A)** according to tumor stage **(B)**. **(C)** Path analysis in patients with LUAD across tumor stage, promoter methylation, mRNA expression, and survival status. The line represents the group; the width of the lines represents the number of patients transferred from one state to another (n=468). **(D)** PPI networks of the *ERO1L* co-expression module. **(E, F)** Functional annotation and pathway enrichment of the *ERO1L* co-expression module. Top 10 terms of GO annotation **(E)** and KEGG pathway **(F)**. *$p < 0.05$.

Co-Expression Module of *ERO1L* in LUAD

In order to investigate proteins that were in close relationship to *ERO1L*, a co-expression analysis of expression data from the TCGA program and Oncomine database was conducted. Via overlapping co-expression results and module mining, 29 proteins were found to be closely related to *ERO1L* (**Figure S2B**). This included ERO1LB, GPX7, GPX8, P4HB, INS, PDIA3, PDIA4, PDIA6, TXNDC5, and ERP44 among others. (**Table S5**). Based on these results, the protein-protein interaction (PPI) network of the *ERO1L* co-expression module was created (**Figure 3D**, **Table S6**). Furthermore, the biological functions of the module were investigated by Gene Otology (GO) analysis (**Figure 3E**) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (**Figure 3F**). Interestingly, it was found that these genes showed strong associations with significant processes such as response to endoplasmic reticulum stress, response to reactive oxygen species, oxidation-reduction process, and glycolytic process. Integrally, this module was shown to be closely related to hypoxia responses as well as the HIF-1 signaling pathway. These might also play a role in shaping the TIME.

Correlations Between *ERO1L* Expression and Immune Cell Markers

To first understand the relationship between *ERO1L* and infiltrating immune cells, a correlation analysis across *ERO1L* and markers for immune cells was performed. These biomarkers are widely used for the purpose of immune cell characterization (**Table 2**). *ERO1L* expression showed strong correlations with markers for infiltrating lymphocytes including regulatory T cells (T_{regs}), exhausted T cells, macrophages, tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and cancer-associated fibroblasts (CAFs), thus indicating infiltrations of immune-suppressive cells are mediated by *ERO1L* signaling (**Figure 4A**). Interestingly, *ERO1L* expression was shown to be positively correlated with the phenotype of M2-type macrophages while negatively correlated with the phenotype of M1-type macrophages. This implies that overexpression of *ERO1L* could indicate the polarization of M1-type to M2-type macrophage (**Figure 4B**).

ERO1L Mediated Immune-Suppressive Tumor Microenvironment Shaping

To confirm whether *ERO1L* expression impacts the TIME, the coefficients of *ERO1L* expression and TIME infiltrations were calculated in the Tumor IMune Estimation Resource 2.0 (TIMER 2.0) database. In relation to tumor-infiltrating lymphocytes, it was found that immune-active cells including B cells (Spearman's ρ=-0.250, p <0.001), $CD8^+$ T cells (Spearman's ρ=-0.299, p <0.001), and NK cells (Spearman's ρ=-0.258, p <0.001) correlated negatively with *ERO1L* expression. After adjustments to account for tumor purity, immune-suppressive cells CAFs (Spearman's ρ=0.286, p <0.001) and MDSCs (Spearman's ρ=0.423, p <0.001) were shown to be positively correlated with *ERO1L* expression (**Figure 4C**).

As a positive correlation was observed between *ERO1L* and $CD4^+$ T cells (Spearman's ρ=0.199, p <0.001), intrinsic $CD4^+$ T cell heterogeneity was further investigated *via* analysis of single-cell sequencing data from dataset GSE99254. Dimensional reduction analysis (t-SNE) applied to the expression data showed that *ERO1L* was highly expressed in most $CD4^+$ T cell clusters. This was consistent with our previous findings (**Figure 4D**). The clusters of CD4-CTLA4 (cluster C9) and CD4-CXCL13 (cluster C7), representing suppressive T_{regs} and exhausted T cells respectively, showed the highest *ERO1L* expression levels. To further confirm *ERO1L* expression across infiltrating cells in TIME, single-cell sequencing data from four projects (GSE7670, GSE31210, GSE32863, and GSE19188) was analyzed. Results showed that *ERO1L* expression closely correlated with infiltrating cell levels including B cells, T cells, NK cells, endothelial cells, macrophages, monocytes, MDSCs, and CAFs (**Figure 4E**, **Figure S2C**). *ERO1L* was more closely associated with the phenotype of a M2 macrophage than a M1 macrophage, which was consistent with our previous findings.

Of note, MDSCs are known to play a key role in immunosuppression in various cancer types. In recent years, increasing evidence has highlighted MDSCs as a major driver behind the immunosuppressive tumor microenvironment. As C/EBPβ and c-Rel have been implicated in MDSC expansion, C/EBPβ and c-Rel expressions were examined. Consistent with previous findings, it was identified that both C/EBPβ and c-Rel were significantly positively correlated with *ERO1L* expression (C/EBPβ: Spearman's ρ=0.144, p <0.001; c-Rel: Spearman's ρ=0.201, p <0.001). This supports the notion that *ERO1L* signaling potentially results in the accumulation of functional MDSCs (**Figure S2D**). Based on the strong correlation observed between *ERO1L* and MDSCs, a survival analysis was performed by constructing a Cox proportional hazards model according to expression profiles of *ERO1L* and MDSC (**Figure 4F**). Results revealed that patients exhibiting low levels of both *ERO1L* and MDSCs experienced a significantly better OS in comparison to those with simultaneously high levels of *ERO1L* and MDSCs (HR:1.55, 95% CI: 1.12-1.84, log-rank p <0.001). This indicates that the combination of high levels of *ERO1L* and MDSC expression can predict poor prognoses in patients with LUAD. In tumors where the high expression level of *ERO1L* was a result of copy number variations including gain and amplification compared with deletion or normal diploid, significant differences were also noted. In specific cases there a decrease in $CD8^+$ T cells and an increase of CAFs and macrophages (**Figures S2E, F**). Taken together, these results indicate that *ERO1L* overexpression is closely related to infiltration of immune-suppressive cells and the deficiency of immune-active cells, thus shaping an immunosuppressive TIME.

ERO1L Overexpression Can Potentially Predict Immunotherapy Resistance

Based on the notion that *ERO1L* overexpression shaped an immune-suppressive TIME, it was hypothesized that high levels of *ERO1L* might also lead to immunotherapy resistance. Given that the MC-38 cell line was sensitive to ICI treatment

TABLE 2 | Correlations between *ERO1L* and gene markers of infiltrating immune cells.

Cell Type	Gene Marker	Without Adjusted		Purity Adjusted	
		Correlation	*p*-value	Correlation	*p*-value
B cell	CD19	-0.065	0.140	-0.140	**
	CD20	-0.119	**	-0.147	**
	CD79A	-0.023	0.606	-0.083	0.067
	CD79B	-0.103	*	-0.182	***
	MS4A1	-0.108	*	-0.183	***
$CD8^+$ T cell	CD8A	0.098	0.262	0.063	0.163
	CD8B	0.046	0.296	0.014	0.761
Th1	IL-2	-0.016	**	-0.148	***
Th2	IL-4	-0.136	**	-0.138	**
	IL-5	-0.012	0.788	-0.001	0.981
T_{reg}	FOXP3	0.123	**	0.093	*
	CCR8	0.175	***	0.168	***
	CD25	0.312	***	0.313	***
	IL7R	0.140	**	0.114	*
T cell exhausted	PD-1	0.133	**	0.100	*
	CTL4	0.107	*	0.075	0.097
	TIM3	0.194	***	0.173	***
	LAG3	0.145	***	0.123	**
DC	CD1C	-0.235	***	-0.269	***
	CD141	-0.065	0.143	-0.081	0.073
Macrophage	CD68	0.241	***	0.224	***
	CD11b	0.152	***	0.137	**
M1	NOS2	0.070	0.112	0.043	0.345
	ROS	-0.096	*	-0.117	**
	IL-12B	-0.179	***	-0.213	***
	HLA-DR	-0.069	0.117	-0.108	*
M2	ARG1	0.018	0.676	0.020	0.664
	MRC1	0.046	0.295	0.027	0.552
	CD68	0.241	***	0.224	***
	CD163	0.256	***	0.248	***
	CD204	0.181	***	0.156	***
TAM	HLA-G	0.154	***	0.131	**
	CD80	0.130	**	0.109	*
	CD86	0.193	***	0.178	***
	CD11b	0.152	***	0.137	**
Monocyte	CD14	0.183	***	0.173	***
	CD16a	0.315	***	0.308	***
	CD16b	0.245	***	0.246	***
MDSC	CD11b	0.152	***	0.137	**
	CD33	0.021	0.639	-0.005	0.905
PMN-MDSC	CD15	0.267	***	0.259	***
M-MDSC	CD14	0.183	***	0.173	***
CAF	FSP1	0.308	***	0.318	***
	FAP	0.314	***	0.314	***
	PDGFRα	0.158	***	0.144	**
	PDGFRβ	0.086	0.052	0.058	0.200
	αSMA	0.105	*	0.081	0.072

*TAM, tumor associated macrophage; MDSC, myeloid-derived suppressor cell; PMN-MDSC, polymorphonuclear myeloid-derived suppressor cell; M-MDSC, monocytic myeloid-derived suppressor cell; CAF, cancer-associated fibroblast; Cor., R value of Spearman's correlation. *p < 0.05; **p < 0.01; ***p < 0.001.*

while the LLC and A549 cell lines were relatively insensitive to ICI treatment, we first performed Western blotting to examine the expression levels of ERO1L protein across these cell lines. Results showed that ERO1L protein was overexpressed in the LLC and A549 cell lines while downregulated in the MC-38 cell line (**Figure S3A**), which was consistent with our hypothesis that overexpression of *ERO1L* might be associated with resistance to ICI treatment.

To further investigate this hypothesis, the stromal and immune cell infiltration levels were analyzed within $ERO1L^{low}$ and $ERO1L^{high}$ samples using ESTIMATE software. Low expression of *ERO1L* was accompanied with a higher abundance of stromal cells and immune cells in comparison to overexpression of *ERO1L*, which was associated with a significantly higher Estimate score. This suggested that an immune-inflamed TIME that may well be susceptible to immunotherapy (**Figure 5A**). To explore this issue in more detail, the Tumor Immune Dysfunction and Exclusion (TIDE) score was used. This score is a computational framework designed to evaluate the potential of tumor immune escape

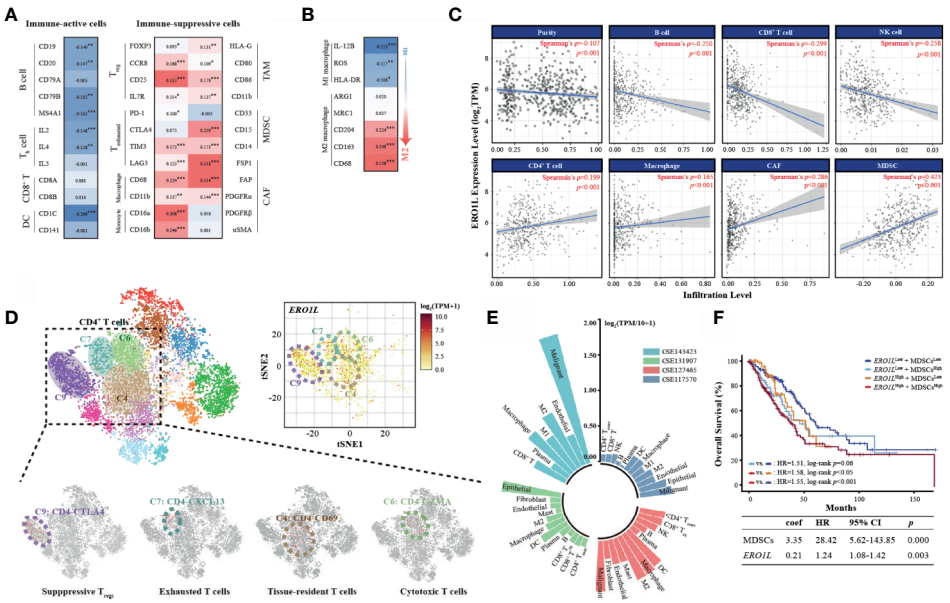

FIGURE 4 | Overexpression of *ERO1L* shapes an immune-suppressive tumor microenvironment. **(A)** Correlations between *ERO1L* and the immune cell markers. **(B)** *ERO1L* expression correlates with macrophages polarization. **(C)** *ERO1L* expression was significantly negatively correlated with infiltrating levels of B cells, CD8+ T cells, and NK cells and significantly positively correlated with infiltrating levels of CD4+ T cells, macrophages, CAFs, and MDSCs. **(D)** The intrinsic heterogeneity of $CD4^+$ T cells according to *ERO1L* expression as determined *via* single-cell sequencing data. Each dot corresponds to a single cell and is colored according to the cell cluster. The color density indicates the expression of *ERO1L*. **(E)** Summary of four single-cell sequencing datasets according to correlations with *ERO1L*. Different datasets are labeled in different colors. **(F)** The Cox proportional hazards model is constructed according to expressions of *ERO1L* and MDSC. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

and is a surrogate biomarker to predict response to immunotherapy. TIDE scores showed that the $ERO1L^{low}$ group had a significantly higher response rate (86.0%) in comparison to the $ERO1L^{high}$ group (31.0%) (**Figures 5B, C**). It was also observed that the $ERO1L^{high}$ group scored high in MDSCs (p <0.001) and immune dysfunction (p <0.001), while scored low in $CD8^+$ T cells (p <0.001) in comparison with the $ERO1L^{low}$ group (**Figure 5D**). To validate these results, the MCP counter was applied to quantify the different immune cell populations within the two groups. In agreement with our results, *ERO1L* overexpression scored low in NK cells (p <0.001), myeloid dendritic cells (p <0.001), neutrophils (p <0.001), and endothelial cells (p <0.001). *ERO1L* overexpression scored high in B lineage (p =0.004), monocyte lineage (p =0.020), and fibroblast (p <0.001; **Figure S3B**). These results suggest that *ERO1L* is in fact a biomarker with potential applications in the prediction of immunotherapy response in patients with LUAD.

Mechanisms Underpinning an *ERO1L*-Induced Immune-Suppressive Tumor Microenvironment

Gene set enrichment analysis (GSEA) was performed in order determine whether the transcriptional signature produced by *ERO1L* overexpression was significantly related to other previously studied conditions. By using hallmark gene sets and all curated gene sets as references, GSEA was performed between the $ERO1L^{low}$ group and $ERO1L^{high}$ group in patients from the TCGA cohort. The global expression changes produced in LUAD patients were positively correlated with the signatures of hypoxia (NES =2.02; FDR q-value =0.0) and VEGF (NES =2.27; FDR q-value =0.0; **Figure 6A**). Moreover, GSEA also revealed that the gene signatures of the JAK-STAT (NES =1.65, FDR q-value =0.0) and NF-κB (NES =2.03, FDR q-value =0.0; **Figure 6B**) signaling pathways were commonly enriched when *ERO1L* signaling was upregulated. The expression levels of the components involved in the two pathways were examined, including JAK1, JAK2, STAT1, STAT2, STAT3, NF-κB1, NF-κB2, RelA, RelB, and c-Rel. Consistent with previous findings, there were significant correlations observed between *ERO1L* overexpression and the aforementioned components (**Figure 6C**).

As the JAK-STAT and NF-κB pathways have previously been reported to play a role in increasing the secretion of immune-suppressive factors, we further explored whether *ERO1L* expression could affect the pattern of cytokines and chemokines secreted by tumor cells and infiltrating immune cells, which play a role in shaping TIME. Via TCGA expression profiling analysis, it was identified that cytokines and chemokines secreted by tumors (such as CSF-1, IL-1β, and IL-6), which have been reported to recruit immune-suppressive cells including MDSCs, TAMs, and CAFs, were positively correlated with overexpression of *ERO1L* (**Figure 6D**). Moreover, it was also identified that immune-suppressive cytokines and chemokines (including IL-10, TGF-β, MMP2, MMP9, and VEGF), which are known to be mostly secreted by immune-

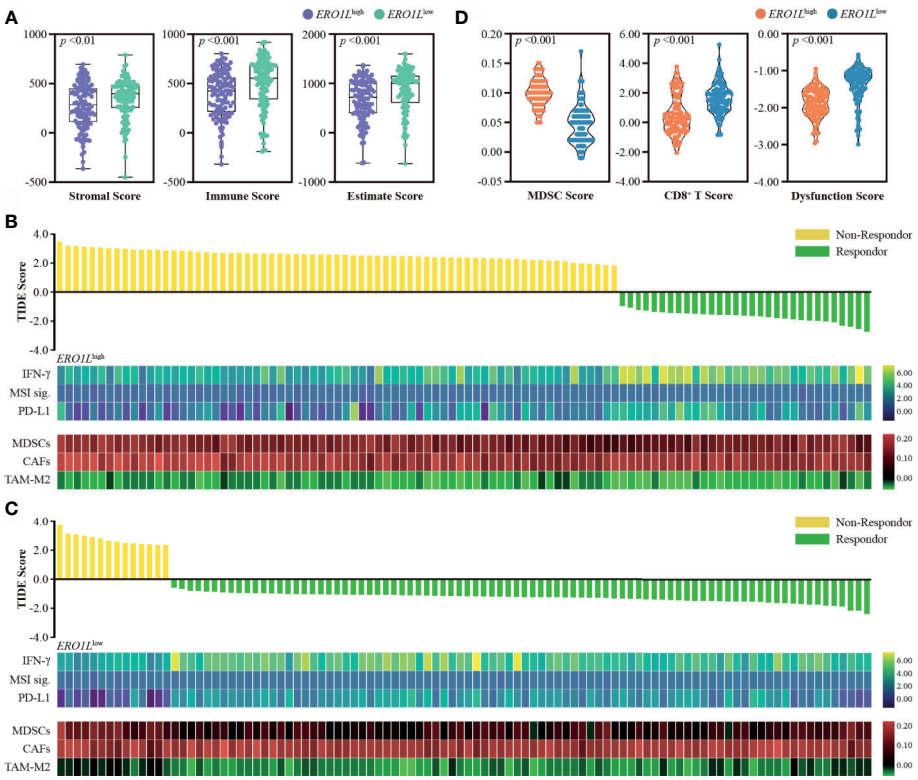

FIGURE 5 | Overexpression of *ERO1L* predicts resistance to immunotherapy. **(A)** Boxplot showing stromal, immune, and Estimate scores within *ERO1L*^{high} and *ERO1L*^{low} groups. **(B, C)** Prediction of immunotherapy response using the TIDE computational framework. Other biomarkers for immunotherapy were also assessed. These included: IFN-γ, MSI signatures, PD-L1, MDSCs, CAFs, and TAM-M2. **(D)** Violin plot showing MDSCs, CD8$^+$ T, and dysfunction scores within the *ERO1L*^{high} and *ERO1L*^{low} groups. Groups are labeled in different colors according to their level of *ERO1L* expression.

suppressive cells, were simultaneously in a positive correlation with overexpression of *ERO1L*. Taken together, these data suggest a potential mechanism for *ERO1L*-associated immune-suppressive TIME (**Figure 6E**).

DISCUSSION

Here, we report a study depicting the biological landscape of *ERO1L* in LUAD. *ERO1L* expression is significantly higher in lung adenocarcinomas in comparison to adjacent normal tissues and is closely related to the prognoses of patients with LUAD. High expression levels of *ERO1L* are associated with a poor prognosis of patients with LUAD. Previous studies have reported that the overexpression of *ERO1L* promoted proliferation, migration, and invasion in pancreatic cancer as well as breast cancer by activating the Wnt/catenin pathway. In this study, overexpression of *ERO1L* was closely associated with infiltrating of immune-suppressive cells and deficiencies in immune-active cells. Therefore, we propose that *ERO1L* functions as an oncogenic factor by inducing an immune-suppressive TIME.

Although *ERO1L* is relatively poorly studied in immunology, molecular studies have investigated the biological functions of the ERO1L protein. This protein is an oxidase in the endoplasmic reticulum which regulates hypoxia-induced oxidative protein folding. Its expression can be induced by hypoxia, which is a common feature of cancers contributing to tumor metastasis, angiogenesis, expansion of tumor-initiating cell, chemoresistance, and genomic instability *via* the regulation of hypoxia-inducible factors such as HIF-1α and HIF-2α. Taking together, these results indicate that *ERO1L* may potentially regulate tumor progression through HIF signaling pathways. In this study, we found that the co-expression module of *ERO1L* took part in oxidation-reduction, glycolytic, and hypoxia. This finding is consistent with previous data. Moreover, hypoxia has also been shown to be an important barrier to effective cancer treatment. We propose that overexpression of *ERO1L* is indicative of a hypoxic TIME, which could potentially confer poor prognoses in patients with LUAD.

ERO1L overexpression is closely associated with the infiltration of immune-suppressive cells including MDSCs, TAMs, and CAFs. This leads to an immunosuppressive TIME. MDSCs are derived from bone marrow and have an inhibitory effect on the immune system. They play an important role in tumor immunosuppression, tumor angiogenesis, drug resistance, and tumor metastasis (24). What's more, MDSCs can produce NO and ROS which can nitrate chemokines and block entry of CD8$^+$ T cells to tumors (25). MDSCs have been reported to

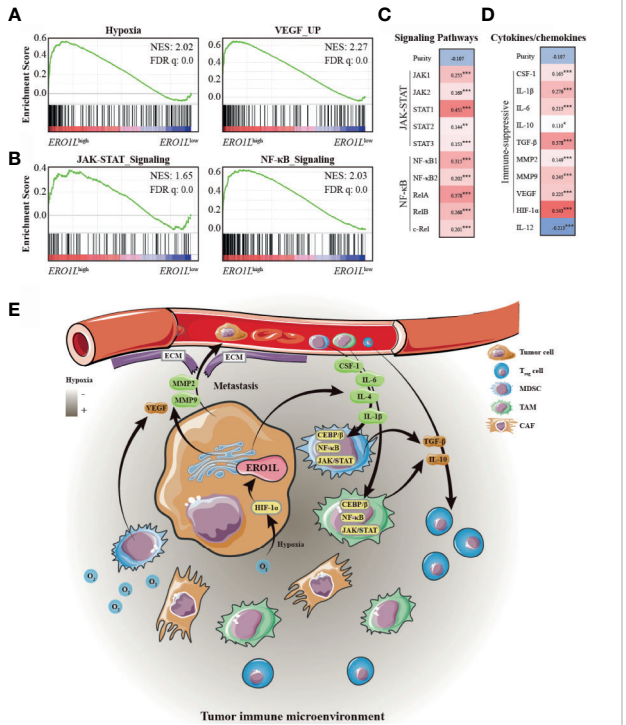

FIGURE 6 | Mechanisms underlying an *ERO1L*-associated immune-suppressive tumor microenvironment. **(A)** GSEA of *ERO1L*^{high} expressing profiles using a hypoxia signature (NES =2.02, q =0.0) and a VEGF upregulated signature (NES =2.27, q =0.0). **(B)** GSEA of *ERO1L*^{high} expressing profiles using an upregulated JAK-STAT signature (NES =1.65, q =0.0) and an upregulated NF-κB signature (NES =2.03, q =0.0). **(C)** Confirmation of gene expression changes in the JAK-STAT and NF-κB signaling pathways. **(D)** Correlations between *ERO1L* overexpression, cytokines, and chemokines patterns in the tumor immune microenvironment. **(E)** Schematic showing the *ERO1L*-induced immune-suppressive tumor immune microenvironment. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

produce immune-suppressive cytokines including IL-10 and TGF-β, which induce T_{regs} and affecting NK cells (26, 27). Furthermore, MDSCs could eliminate the key nutrition factors needed for T cell proliferation *via* the depletion of L-arginine (28), sequestering L-cysteine (29), or reducing local tryptophan levels due to the activity of indoleamine 2,3 dioxygenase (30). What's more, recent studies have demonstrated that MDSCs were highly significantly associated with poor OS and PFS in gastrointestinal cancer, hepatocellular carcinoma, lung cancer, and multiple myeloma (31).

TAMs generally display as M2 phenotype macrophages which are devoid of cytotoxic activity, produce growth factors for cancer cells, and have immune-suppressive activity (32). TAMs preferentially localize in the hypoxic areas of tumors, where they promote the expression of the transcription factor HIF-1α. This transcription factor induces the transcription of various elements including VEGF, basic fibroblast growth factor, platelet-derived growth factor, and prostaglandin E2, which is associated with angiogenesis (33). TAMs have potential to produce enzymes and proteases such as MMPs including MMP2 and MMP9 which regulate the degradation of the extracellular matrix (ECM). ECM disruption by TAMs facilitates tumor cell spreading and metastasis (34). What's more, TAMs also contribute to immune-suppression in the TIME *via* inhibition of IL-12. On the contrary, TAMs promote the secretion of IL-10 and TGF-β, which block T cell proliferation, suppress cytotoxic T lymphocyte (CTL) responses, and activate T_{regs} (35). Clinical studies have demonstrated a strong association between poor survival and increased macrophage density in thyroid, lung, and hepatocellular cancers (36, 37). Similarly, our research proved that in tumors with high expression levels of *ERO1L* are positively associated with the secretion of cytokines and enzymes such as HIF-1α, MMPs, IL-10, TGF-β, and VEGF.

Recently, immune checkpoint inhibitors have led to a paradigm shift in treatment for patients with non-small cell lung cancer (NSCLC). However, the efficacy of these treatments is less than 50%. The clinical responses of ICI are reported to be unfavorable because of the low tumor mutation burden, low PD-L1 expression, and the noninflamed TIME. Based on the results presented in this study, we hypothesized that activation of *ERO1L* signaling could recruit immune-suppressive cells and shape an immune-suppressive TIME and thus conferring resistance to ICI treatment. We propose that *ERO1L* overexpression is an effective biomarker for noninflamed TIME. However, our conclusions were mainly summarized based on the public datasets; further studies based on grafted tumors and patients' samples are highly needed.

CONCLUSION

In summary, our study provides clear insight into the potential role of *ERO1L* in tumor immunology. Our study also suggests the potential prognostic value of *ERO1L* in patients with LUAD. We described that overexpression of *ERO1L*, indicates a hypoxic environment and shapes an immune-suppressive TIME through the recruitment of immune-suppressive cells and inhibition of immune-active cells. High levels of *ERO1L* may be indicative of resistance to immunotherapy. *ERO1L* was shown to associated with cytokine and chemokine patterns in the TIME, which were resulted from activations of JAK-STAT and NF-κB signaling pathways. These findings suggest a potential immune-based anti-tumor strategy *via* the inhibition of *ERO1L* to clear tumor microenvironment infiltrates.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

JW and HB conceived, designed, and supervised the study. YQ, ZM and XZ collected and visualized the data. LL, CW, and SL

performed organoid-related experiments. LL, CW, and PX analyzed the data. LL and CW interpreted findings and wrote the first draft of the manuscript. JW and HB revised and edited the manuscript. All authors approved the final version of the manuscript. JW is the guarantor of this study and accepts full responsibility for the work, had access to the data, and controlled the decision to publish. The corresponding authors attest that all listed authors meet authorship criteria and that no other person meeting the criteria has been omitted. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.677169/full#supplementary-material

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REVIEW
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Personalized Neoantigen-Pulsed DC Vaccines: Advances in Clinical Applications

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In the past few decades, great progress has been made in the clinical application of dendritic cell (DC) vaccines loaded with personalized neoantigens. Personalized neoantigens are antigens arising from somatic mutations in cancers, with specificity to each patient. DC vaccines work based on the fundamental characteristics of DCs, which are professional antigen-presenting cells (APCs), responsible for the uptake, processing, and presentation of antigens to T cells to activate immune responses. Neoantigens can exert their antitumor effects only after they are taken up by APCs and presented to T cells. In recent years, neoantigen-based personalized tumor therapeutic vaccines have proven to be safe, immunogenic and feasible treatment strategies in patients with melanoma and glioblastoma that provide new hope in the treatment of cancer patients and a new approach to cure cancer. In addition, according to ClinicalTrials.gov, hundreds of registered DC vaccine trials are either completed or ongoing worldwide, of which 9 are in early phase I, 191 in phase I, 166 in phase II and 8 in phase III. Hundreds of clinical studies on therapeutic tumor vaccines globally have proven that DC vaccines are stable, reliable and very safe. However, in this process, many other factors still limit the effectiveness of the vaccine. This review will focus on the current research progress on personalized neoantigen-pulsed DC vaccines, their limitations and future research directions of DC vaccines loaded with neoantigens. This review aims to provide a better understanding of DCs biology and manipulation of activated DCs for DCs researchers to produce the next generation of highly efficient cancer vaccines for patients.

Keywords: personalized neoantigen, DC vaccine, tumor, immunotherapy, clinical applications

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INTRODUCTION

Malignant tumors are still an acute threat for people worldwide and the incidence and mortality from cancer are still rapidly growing. GLOBOCAN showed an estimated 19.3 million new cases and 10 million cancer deaths worldwide in 2020; at the same time, an estimated 28.4 million new cancer cases are projected to occur in 2040 (1). Therefore, it is still difficult to find proper and effective ways to fight cancer.

After decades of effort, conventional methods and systems for treating cancer have been developed, including surgery, radiotherapy and chemotherapy alone or in combination.

Surgery is the preferred treatment for most tumors; however, it is a traumatic and local treatment that easily leads to surgical complications. Although radiotherapy is the most suitable method for tumors in all parts of the body, the radiation dose that the body can withstand is limited, and normal cells are also damaged when tumor cells are destroyed. Although chemotherapy is successful for some tumors, such as testicular tumors, it can cause severe side effects, such as hair loss, anemia and organ damage, reducing the patients' quality of life (2, 3). Because of the side effects of conventional treatments, cancer immunotherapy has been developed as a therapeutic method with better tumor targeting, safety, and a lower toxicity.

Cancer immunotherapy relies on the individual's own immune system to recognize and control cancer progression to fight and cure cancer (4). At the same time, cancer immunotherapy has been developed to enhance the antitumor response of the immune system and reduce off-target effects and other serious side effects of other conventional therapies (5). There are five main types of cancer immunotherapy (6):

(i) Immune checkpoint inhibitors, in which the most extensive strategies involve the use of programmed death 1/programmed death ligand 1 blockade (PD1/PD-L1 blockade) and cytotoxic T lymphocyte-associated antigen-4 inhibition (CTLA-4 inhibition). Immune checkpoints are immunosuppressive pathways that regulate the immune response to maintain tolerance and protect the surrounding tissues. This property is used by tumor cells to escape the attack of immune cells, and immune checkpoint inhibitors can inhibit immune checkpoint activity and reactivate the immune response of T cells to the tumor to achieve an antitumor effect (7).

(ii) Cytokines, which contain three main types (interleukins, interferons, and granulocyte–macrophage colony-stimulating factor (GM-CSF)) (8), are the first class of approved immunotherapies for clinical use and have effects *via* stimulating immune cells directly (6, 9).

(iii) T cells and natural killer (NK) cells, in which T cells include engineered T cells and non-engineered T cells such as adoptive tumor infiltrating lymphocyte (TIL) and cultivated T cells. Engineered T cells contain chimeric antigen receptor T cells (CAR-T) and T cell receptor T cells (TCR-T), and CAR-T cells can trigger the death of tumor cells by recognizing the targeted antigens on tumor cells (10), and the antitumor activity of TCR-T cells is mainly stimulated by tumor-associated antigens presented by major histocompatibility complexes (MHCs) (11). NK cells also include engineered NK cells such as CAR-NK cells and many trials are under way.

(iv) Agonistic antibodies, which can specifically bind to receptors on the surface of T cells, triggering intracellular signaling pathways and inducing T cells to function as effectors to kill tumor cells (12).

(v) Cancer vaccines include those based on tumor cell lysates, nucleic acids, and peptides, which contain or can encode neoantigens (13). Neoantigen vaccines are an attractive type of cancer vaccine. In addition to being used separately as vaccines, DNA, RNA, peptide and tumor lysate can also be loaded onto DCs (**Figure 1**). Although DNA can be easily manipulated by molecular engineering, the successful use of the first generation of drug delivery platforms in humans is limited, and they tend to rely more on electroporation and it is also limited by its potential to integrate into the genome (14). On the other hand, there is no potential risk for RNA to integrate into the genome; however, it is still affected by RNase degradation although modification may prolong its half-life (14). In addition, synthetic long peptide (SLP) is easy to store, low toxicity and appropriate adjuvants are required (14). Therefore, when working with DC vaccines, the choice needs to be made between either loading with peptides, RNA, DNA or tumor lysate. Furthermore, DCs pulsed with neoantigens *ex vivo* to treat patients can effectively induce anti-tumor immune responses induced by activated T cells (13). Hundreds of research and clinical trials have been conducted or are underway since the first DC vaccine, sipuleucel-T, was approved for clinical use in 2010 (15). Although the safety of DC vaccines has been demonstrated in several clinical trials, several clinical trials have still failed due to the lack of clear efficacy (16, 17). The emergence of personalized neoantigens that were isolated, identified and selected from the patients' tumors and their entry into the body after loading on DCs *ex vivo* can promote the efficient presentation of neoantigens by DCs to T cells to exert an anti-tumor role (16) (**Figure 2**). In this review, we summarize the progress and clinical application of personalized neoantigen-pulsed DC cancer vaccines.

PERSONALIZED NEOANTIGENS

Neoantigens are a series of peptides with tumor specificity that are present in proliferating tumor cells but not in normal tissues. Therefore, they are different from tumor-associated antigens (TAAs) mostly present in both normal and tumor tissues, which also include viral antigens (18, 19). At the same time, they are derived from viral proteins such as open reading frame-derived epitopes in the viral genome and tumor somatic nonsynonymous genetic alterations, including genomic variant level such as single nucleotide variants (SNVs), insertion-deletions, gene fusion, frame shift mutation and transcriptomic and proteomic variants (20–23). The change in peptide sequence and its spatial structure can result in a stronger affinity for major histocompatibility complexes (MHCs), and therefore, making it more likely to be recognized by T cells to induce antitumor immune responses (24). In general, neoantigens are divided into two subgroups: shared neoantigens and personalized neoantigens (25). Shared neoantigens are common in some tumor types and can be used broadly to treat patients who have the same tumor type and express these antigens; however, there are antigenic differences between different patients and different tumors, limiting the role of shared antigens (26, 27). Unlike shared neoantigens, personalized neoantigens are a class of antigens specific to individual patients and tumors. Since tumors of the same cancer type can vary greatly, personalized treatment with personalized neoantigens is a better way to ensure a response by each cancer type (25).

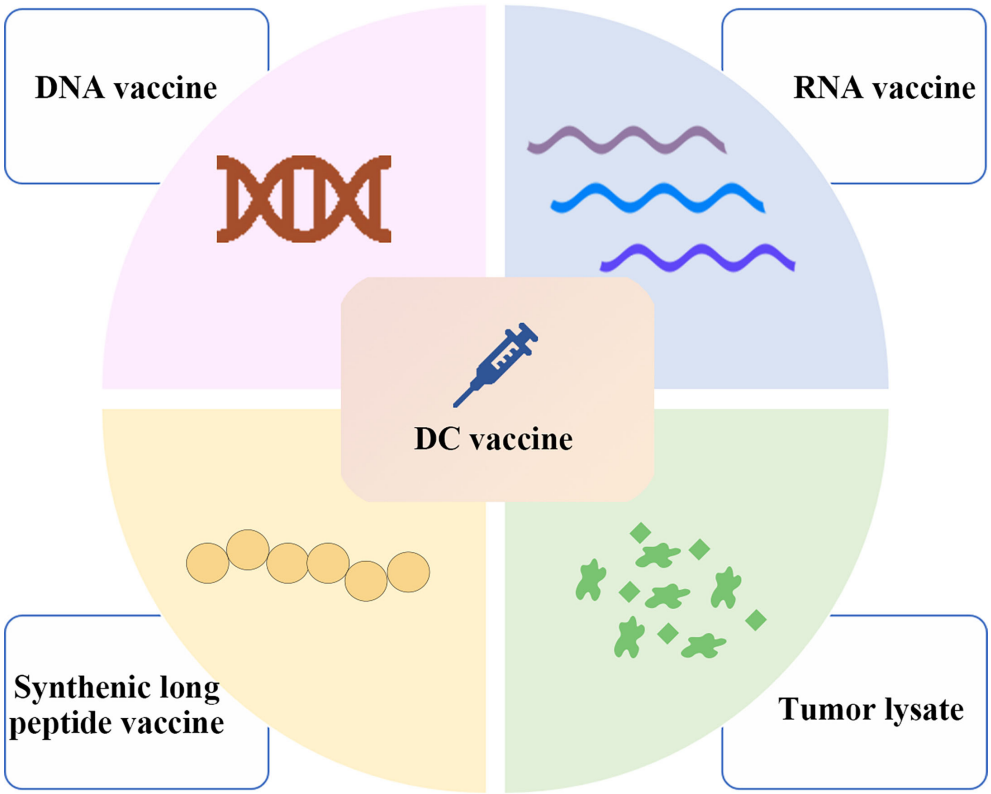

FIGURE 1 | Major types of neoantigen vaccines in clinical research. Neoantigen vaccines mainly include nucleic acid vaccines consisting of DNA and RNA vaccines, synthetic long peptide vaccines and tumor lysate vaccines. In addition to being used separately as vaccines, these neoantigens formulations can also be loaded onto DCs. Therefore, when working with DC vaccines, still the choice needs to be made between loading with either peptides, RNA, DNA or tumor lysate.

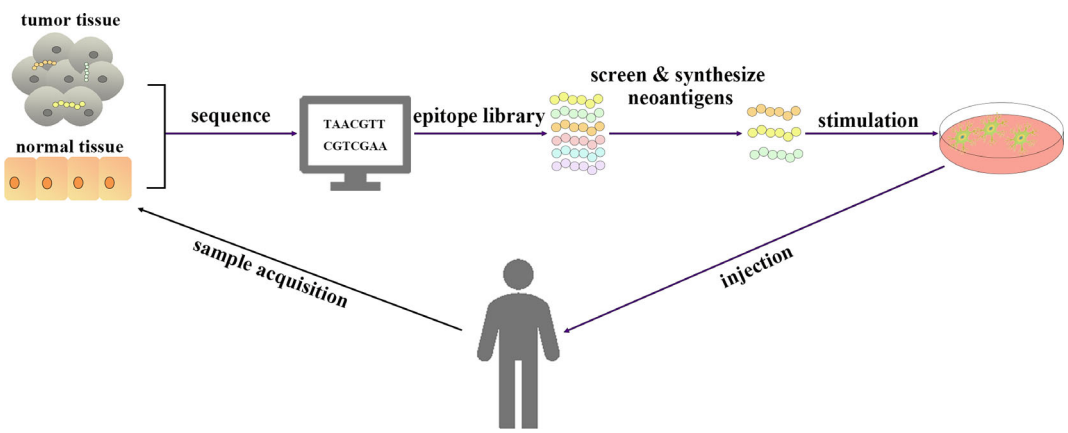

FIGURE 2 | Schematic diagram of personalized neoantigen-pulsed DC vaccines. Tumor tissue and normal tissue of patients were sequenced. The epitope library is processed by bioinformatics methods, from which immunogenic neoantigens are screened and synthesized. The DC vaccines loaded with neoantigens are prepared by using DCs extracted from the peripheral blood of patients and injected into patients.

A series of completed or ongoing clinical trials on personalized tumor neoantigens is listed in **Table 1** according to the data on ClinicalTrials.gov. In the trial conducted by Ott (28), they enrolled 10 patients, 8 of whom displayed a high degree of melanoma-related mutations as expected, and then 13-20 immunizing long peptides were synthesized for each patient. Finally, 6 patients completed the full series vaccinations. No disease recurrence was observed in 4 patients during a median follow-up period of 25

TABLE 1 | The clinical application of personalized neoantigen vaccines.

Tumor types	Phase	Status	Participants	NCT number
Melanoma	Phase I	Completed	20	NCT01970358
Pancreatic Cancer	Phase I	Recruiting	60	NCT03558945
Kidney Cancer	Phase I	Recruiting	19	NCT02950766
Bladder Cancer	Phase I	Recruiting	15	NCT03359239
Pancreatic Cancer	Phase I	Recruiting	20	NCT04161755
Non-Small-Cell Lung cancer	Phase I	Recruiting	20	NCT04487093
Glioblastoma	Phase I	Recruiting	56	NCT02287428
Melanoma	Phase I/II	Recruiting	25	NCT03715985
Bladder Cancer				
Non-Small-Cell Lung cancer				
Melanoma	Phase I	Recruiting	30	NCT04072900
Breast Cancer	Phase II	Recruiting	70	NCT03606967
Small Cell Lung Cancer	Phase II	Not yet recruiting	27	NCT04397003
Diffuse Intrinsic Pontine Glioma	Phase I	Not yet recruiting	30	NCT04749641
Melanoma	Phase II	Active, not recruiting	60	NCT02129075

months (range 20-32 months) after vaccination. Two other patients suffered disease recurrence but also had a complete response after anti-PD1 therapy, which still shows that personalized neoantigen-based vaccines are safe and feasible and could be used in the clinic.

Identification of Personalized Neoantigens

The identification of personalized neoantigens is an important part of tumor immunity therapy to allow personalized neoantigens to have an effect in each patient. The common approach is to compare DNA sequences in tumor tissues with those in normal tissues using high-throughput sequencing technologies (next-generation sequencing, NGS), which is rapid and efficient (29). However, many of the detected DNA mutations are not expressed as they are noncoding mutations or nonsense mutations, which poses new challenges to identify neoantigens (29). With the progress of sequencing technology, a more efficient and feasible sequencing technology with a lower false-negative rate was born: whole-exome sequencing technology (the exome is the protein-encoding part of the genome), which is currently widely used to identify personalized neoantigens (30). The mutant amino acid sequence that can be expressed needs to be translated and processed into short peptide fragments. These also need to be expressed on the cell surface in complex with MHC molecules to be recognized successfully by the immune system (19). Therefore, there are several crucial factors that determine whether a mutation can produce an effective personalized neoantigen: (i) whether the mutated DNA sequence can eventually be expressed and processed into short peptide fragments at the protein level; (ii) the ability of peptides to be presented and their affinity to MHC molecules; (iii) the affinity of the complex formed by the mutant peptides and MHC to TCR (31).

Because of lots of work involved in comparing high-throughput sequencing data, the development of computer simulation experiments or tools has effectively promoted the identification of personalized tumor neoantigens. On the one hand, for different neoantigen sources, there are corresponding computational tools. For single nucleotide variants (SNVs), small insertions and deletions (INDELs), or gene fusion at the genomic variant level, pVAC-Seq, TSNAD, CloudNeo, Tlminer, MuPeXI, Neopepsee, and INTEGRATE-Neo are usually utilized (32–37). For alternative transcript splicing at the transcriptomic variant level, NeoantigenR is widely used (38). On the other hand, these mutations need to be ranked according to their affinity with individual autologous MHC molecules; for this, the tools NetMHC, SMMPMBEC and SMM, among others, are used (39–41). Of the MHC molecule, MHC-I is directly related to neoantigen presentation on tumor cells, and the methods of using the MHC-I molecule to predict neoantigens are relatively mature at present, while $CD4^+$ T cells recognize predicted neoantigens presented by MHC-II molecules. Compared to MHC-I molecules, where the peptide-binding groove is closed at both ends, the binding groove of MHC-II molecules is open at both ends and can deliver longer peptides (11-20 amino acids) (42). However, there is currently a lack of robust and rich databases and effective tools for assessing the interactions between MHC-II molecules and peptides compared with what is available for MHC-I (42). The further development of bioinformatics resources and the use of other cross-disciplinary methods are expected to improve neoantigen identification.

Personalized Neoantigens Manufacturing

Personalized neoantigens are a unique class of neoantigens specifically prepared for each patient; therefore, a rapid, simple, and mature system for the synthesis of personalized neoantigens is needed, as this is the first step of manufacturing neoantigens (43). In addition, the formulation of neoantigens such as buffering agents and surfactants are another crucial factor due to the different compositions and properties of each personalized neoantigen and these other components play important roles in ensuring the solubility and stability of neoantigens (44). The next step is purification. Many systems are used to purify neoantigens such as RP-HPLC and flash-like systems. With the development of new technologies, an increasing number of manual operations have been replaced by automated processes such as auto-sampling systems and ultra-performance liquid chromatography (UPLC) (45), saving time while enhancing the productivity and quality. The last step is lyophilization to make the newly prepared neoantigens easier to transport and store until used. Similarly, as

technology advances, the processing of personalized neoantigens will become faster and more efficient, saving the patient precious time and increasing the effectiveness of cures.

PERSONALIZED NEOANTIGENS-PULSED DC VACCINES

Definition and Types of DCs

Immune cells, which include B cells, T cells, natural killer cells (NK cells) derived from lymphoid stem cells and neutrophils, eosinophils, basophils, and monocytes derived from myeloid progenitor, produce an immune response to resist the invasion of bacteria and viruses, kill tumor cells and maintain the human body's immune balance. DCs, often differentiated from monocytes, are professional antigen-presenting cells and are responsible for efficient uptake, processing and presentation of antigens, which can not only teach naive T cells to become antigen-specific cytotoxic T cells (CTLs) through antigen presentation but also allow for interaction with other immune cells in the body, such as NK cells, T cells and B cells, activating the immune system to recognize and kill tumors (46).

In humans, committed DC precursors (CDPs) in bone marrow are divided into two major subsets of DCs, plasmacytoid DCs (pDCs) and conventional DCs (cDCs), which include two major categories-cDC1 and cDC2 based on their phenotype (47). These cells circulate in the blood and continue to enter the lymphoid organs and peripheral tissues as a supplement to DCs. For pDCs, surface markers mainly include CD123, CD303, CD304, and CD45RA, and they specifically secrete type I interferons (IFN-I) while presenting antigens to T cells and activating T cells (48, 49). For cDC1, surface markers mainly include Cleca9A, XCR1, and CD141, and cDC1 have the ability to cross-present and induce cytotoxic T cell immune responses and can also significantly stimulate the immune response of allogeneic or autologous $CD4^+$ T cells (48, 49). For cDC2, surface markers mainly include CD1c, CD1a, and CD103, which can present soluble antigens but rarely present antigens derived from necrotic cells (48–50). In conclusion, different DCs play different physiological functions in the body, promoting the important role of DCs in immune regulation.

DC Vaccines

The principle of preparing a DC vaccine is simple. The precursor cells of DCs in patients are isolated and cultured *in vitro*, loaded with tumor antigens, and then transferred back into patients. Then, the antitumor effect can be exerted by specific antitumor T cells stimulated by DCs. After nearly 10 years of effort in the field of DC vaccines, in 2000, DC-based immunotherapy was used for the first time in a patient with a primary intracranial tumor. The patient received 3 treatments with an allogeneic MHC class I glioblastoma peptide-pulsed DC vaccine. The trial showed that the DC vaccine was tolerated, and the patient received a positive immune response. However, no objective clinical response was observed (51). In 2010, the United States FDA approved Sipuleucel-T as the first therapeutic DC vaccine for prostate cancer. Sipuleucel-T consists of peripheral blood mononuclear cells (PBMCs), which include APCs, activated *ex vivo* by PA2024, a recombinant protein including mainly prostate-specific antigen and prostatic acid phosphatase (15).

Philip W. Kantoff's group divided 512 patients into two groups at a ratio of 2:1 to receive treatment with Sipuleucel-T and placebo every two weeks by intravenous injection, for 3 treatments in total (52). The results showed that the 36-month survival was 31.7% in the Sipuleucel-T group and 23% in the placebo group. The median survival duration in the Sipuleucel-T group was 25.8 months, an increase of 4.1 months compared with 21.7 months in the placebo group. This revealed that the drug could significantly prolong the survival period, suggesting that the DC vaccine can give patients a survival benefit. Another clinical trial on glioblastoma also showed superior efficacy of a DC vaccine. ICT-107 is an autologous DC vaccine pulsed with 6 different peptides targeting glioblastoma. In a prior phase I study, 21 patients with glioblastoma administered ICT-107 showed good tolerance and in 16 newly diagnosed patients, 6 patients did not show tumor recurrence, which showed that this DC vaccine was well tolerated and possessed antitumor activity (53).

In the following phase IIb trial conducted by Patrick Y. Wen, among HLA-$A2^+$ patients with a matriculated MGMT promoter, progression-free survival (PFS) in the ICT-107 group (24.1 months) was significantly higher than that in the control group (8.5 months) and the patients in the ICT-107 group showed improved immune responses (54). Although many trials have focused on DC vaccines in recent years (**Table 2**), the basis of DC vaccines is the selection of immunogenic antigens to activate the immune system effectively in addition to the maturation of DCs. Because the antigens in each patient's tumor are highly specific, DCs loaded with personalized neoantigens for fusion into therapeutic tumor vaccines are another attractive strategy.

Clinical Trial Progress of Personalized Neoantigen-Pulsed DC Vaccines

Tumor vaccines that rely on neoantigens alone cannot completely eliminate malignant tumors (55). The reason for this is not the neoantigen itself but more because most of the trials used neoantigens to solve the problem of the weak antigenicity of tumor cells but did not solve the problem of immune cell functional defects in cancer patients. Patients with malignant tumors usually have a low level of immune function, and it is difficult to initiate the antitumor immune response *in vivo*. One of the main reasons is that the function of antigen-presenting cells in patients is inhibited, and antigen-activated T cells cannot be effectively presented. Therefore, to achieve good clinical efficacy, immunotherapy should not only solve the problems related to antigens but also the problems of immunosuppression in tumor patients. In other words, when many tumor-specific antigens are injected into the body, it is necessary to ensure that they are efficiently taken up and presented by the body's antigen-presenting cells and that a sufficient number of effector T cells are activated.

In 2015, the first personalized neoantigen-loaded DC vaccine began testing in a phase I clinical trial (56). They enrolled 3 melanoma patients with stage III resected cutaneous melanoma and treated them with ipilimumab. Then, they identified somatic

TABLE 2 | The clinical application of DC vaccines.

Tumor types	Phase	Status	Participants	Source of DCs	NCT number
Breast Cancer	Phase I/II	Completed	10	/	NCT02018458
Breast Cancer	Phase I/II	Completed	44	/	NCT01042535
Unspecified Adult Solid Tumor					
Breast Cancer	Phase I	Completed	31	monocytes	NCT00978913
Malignant Melanoma					
Colorectal Cancer	Phase I	Completed	6	monocytes	NCT01671592
Lung Cancer	Phase II	Completed	32	white blood cells	NCT00103116
Prostate Cancer	Phase II	Completed	13	/	NCT00970203
Hematological Malignancies	Phase I/II	Completed	10	/	NCT02528682
Gastric Cancer	Phase I/II	Recruiting	45	/	NCT04567069
Colorectal Cancer	Phase I	Recruiting	12	/	NCT03730948
Glioblastoma	Phase II/III	Recruiting	60	/	NCT03548571
Breast Cancer	Phase I	Active, not recruiting	15	/	NCT02063724

mutations from their own surgically excised tumors by whole-exome sequencing and computer-simulated epitope prediction to screen for suitable neoantigens. Furthermore, 7 neoantigens selected from each patient were loaded with DCs isolated from PBMCs, cultured *ex vivo*, and then intravenously injected into the patients for a total of three treatments. After the treatments, an enhanced immune response triggered by T cells was observed, while three patients were all surviving and no autoimmune adverse reactions were observed, which showed that DC vaccines pulsed with personalized neoantigens was safe and reliable.

In another trial conducted by Ding's group in 2020, they demonstrated for the first time the activity of a personalized neoantigen-pulsed DC vaccine in patients with advanced NSCLC (57). In their study, they enrolled 12 patients with advanced lung cancer and 13-30 peptide-based personalized neoantigens were isolated and identified from each patient's tumor tissue. At the same time, PBMCs were derived from each patient, DCs were separated, then DCs were pulsed with the corresponding selected neoantigens to form a personalized neoantigen-pulsed DC vaccine to treat patients. Their study showed a 25% objective response rate, while a 75% disease control rate was observed after treatment of a personalized neoantigen-pulsed DC vaccine. In addition, only low-grade and transient side effects were observed, which also demonstrated that the vaccine was safe and able to induce specific T cell immune response. In particular, a patient with metastatic lung cancer whose main metastases were in bone, pelvis, and inferior vena cava lymph nodes failed to show a tumor response after three treatments. Then, he received personalized neoantigen-pulsed DC vaccine treatment, and after 5 doses of this vaccine, almost no metastatic lymph nodes and shrinking pelvic lesions were observed. There was a 29% reduction in overall tumor lesions, which showed a good therapeutic effect of the vaccine.

In Sarivalasis's paper published in 2019, they present another phase I/II trial that uses personalized peptides, including tumor-specific neoantigens and TAAs derived from patients, and pulses them into DCs isolated from autologous monocytes (58). They will acquire the tumor specimens from each patient for NGS analysis. Then they will analyze the data to generate personalized databases, and up to 10 will be selected per patient by verifying the immune response of the candidate peptides to T cells isolated from the patient. This trial will investigate the feasibility and safety of a personalized neoantigen-loaded DC vaccine in patients with ovarian cancer and evaluate overall survival (OS) progression time and disease-free survival at 12, 24, and 36 months. This trial is the first of its kind to test a personalized neoantigen-pulsed DC vaccine in ovarian cancer patients. We look forward to its expected efficacy in a clinical trial, providing additional strong evidence of the efficacy and safety of a personalized neoantigen-pulsed DC vaccine and bringing benefits to patients.

In addition, according to ClinicalTrials.gov, there are several clinical trials around personalized neoantigen-loaded DC vaccines under way that are in phase I (**Table 3**). Although these trials are underway, the fact that they have been carried out only in the last decade shows that they are still forward-looking and innovative.

PERSONALIZED NEOANTIGEN-PULSED DC VACCINES IN COMBINATION WITH OTHER THERAPIES

The combination of personalized neoantigen-pulsed DC vaccines with other strategies, such as chemotherapy and immune checkpoint inhibitors, is another attractive approach to enhance the tumor therapeutic vaccine efficacy. Chemotherapy is considered an immunotherapy partner to improve immunotherapy efficacy by enhancing antigen production and presentation, and inducing T cell immune response, although it still has several side effects (59). In a trial conducted by Batich and colleagues (60), cytomegalovirus antigen pp65 was found to be present in glioma cells instead of surrounding normal tissues. Then, they used a pp65-pulsed DC vaccine combined with dose-intensified temozolomide, which is a chemotherapeutic drug to treat glioma. As expected, the median PFS was 25.3 months and OS was 41.1 months, and both were much higher than the statistical median survival of patients (less than 15 months) with newly diagnosed glioma.

Immune checkpoints exert strong immunosuppressive effects to block the antitumor immune response; thus, neoantigen vaccines combined with immune checkpoint inhibitors, which involve mainly specific monoclonal antibodies such as anti-PD-1, anti-PD-L1, and anti-CTLA-4 antibodies, are thought to generate strong a T cell immune response to kill tumors (7, 61, 62). In Sahin's trial, neoantigen-specific T cells were $PD1^+$ and after the neoantigen vaccine, PD-L1 upregulation was observed (63). Then, anti-PD1 treatment was applied after the neoantigen vaccine, and a complete

TABLE 3 | The clinical application of personalized neoantigen-pulsed DC vaccines.

Tumor types	Phase	Status	Participants	Source of DCs	NCT number
Breast Cancer	Phase I	Completed	9	monocytes	NCT04879888
Triple Negative Breast Cancer	Phase I	Recruiting	5	/	NCT04105582
Gastric Cancer Hepatocellular Carcinoma Non-Small-Cell Lung Cancer Colon Rectal Cancer	Phase I	Recruiting	80	/	NCT04147078
Pancreatic Adenocarcinoma	Phase Ib	Recruiting	12	PBMC	NCT04627246
Non-Small-Cell Lung Cancer	Phase I	Recruiting	6	monocytes	NCT04078269
Advanced Biliary Tract Tumor	Phase I/II	Recruiting	40	/	NCT02632019
Non-Small-Cell Lung cancer	Phase I	unknown	20	/	NCT02956551
Liver Cancer	Phase I	unknown	24	/	NCT03674073
Non-Small-Cell Lung cancer	Phase I	unknown	30	/	NCT03871205
Glioblastoma	Phase I	Enrolling by invitation	10	/	NCT03914768
Colorectal Cancer	Phase I/II	Active, not recruiting	25	/	NCT01885702
Non-Small-Cell Lung cancer	Phase I/II	Not yet recruiting	20	peripheral blood	NCT03205930

response to the neoantigen vaccine was observed. In a phase I trial, the combination of MART-1 peptide-pulsed DCs and tremelimumab, an anti-CTLA-4 antibody, was used for 16 patients with melanoma and they acquired a higher durable objective tumor response rate than treatment alone (64). In addition, a trial conducted by Ding showed that an enlarged tumor was still observed in a patient with lung cancer after treatment with a personalized neoantigen-pulsed DC vaccine (57). When nivolumab, an anti-PD-1 antibody, was combined with this DC vaccine, the patient's tumor became cavitated, which demonstrated the superiority of combination therapy for cancer.

FACTORS THAT LIMIT THE EFFECTIVENESS OF PERSONALIZED NEOANTIGEN-PULSED DC VACCINES

Although the current clinical application shows efficacy, personalized tumor neoantigen-pulsed DC vaccines are still limited in several aspects. (i) The selection of neoantigens: The sequencing and screening of tumor neoantigens requires individual detection and analysis for each patient's tumor, which is a complex and time-consuming process. Additionally, the manufacture of neoantigens requires a better manufacturing conditions to ensure the consistency of neoantigens (65). As a result, the development and wide application of advanced technology are urgently needed. It is believed that the time and production cost of this process will be greatly reduced in the near future. (ii) The source and maturation conditions of DCs: DCs applied in personalized neoantigen-pulsed DC vaccines are also individualized, and it is necessary to extract DCs from each patient for separate culture. In addition, mature DCs are needed to enhance antigen processing, presentation and stimulate B and T cells. Antigens, cytokines such as GM-CSF and other factors such as LPS could stimulate DCs maturation. This process still has problems such as the intensive labor required for the *ex vivo* culture process and the skill required for inducing DC maturation. Thus, in future studies, efforts are needed to optimize *ex vivo* culture while inducing mature and high-quality DCs (66–69). (iii) The efficiency of DC migration: DCs injected back into patients should migrate to the lymphoid organs to stimulate T cells to achieve effective immune responses, and some proinflammatory cytokines, such as prostaglandin E_2 (PGE_2), could promote the migration of DCs to some extent (70–72). However, selective migration of DCs and their residence in nonlymphoid and lymphoid organs are tightly regulated events. The molecular control mechanisms need to be elucidated in future studies to lay the foundation for improving the stimulation conditions of DC vaccines in clinical trials.

CONCLUSION

Personalized tumor neoantigens are highly specific to individuals, and tumor vaccines targeting neoantigens can effectively induce T cells to produce a strong immune response against tumors. However, the key to the effectiveness of personalized tumor neoantigens is that they can be efficiently taken up and processed by APCs and delivered to T cells to induce an antitumor immune response. However, the function of antigen-presenting cells in patients with malignant tumors is usually inhibited. Therefore, the treatment of patients with DC vaccines loaded with neoantigens can specifically target the tumor and ensure that DCs can exert their efficacy to the maximum extent. An increasing number of research and clinical trials are currently underway, promising to offer new hope to patients with solid tumors.

AUTHOR CONTRIBUTIONS

LY and LT conceptualized the study. LT and RZ finished the manuscript and figures, and XZ helped LT collect the related paper. All authors contributed to the article and approved the submitted version.

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The Clinical Application of Neoantigens in Esophageal Cancer

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Esophageal cancer (EC) is a common malignant tumor with poor prognosis, and current treatments for patients with advanced EC remain unsatisfactory. Recently, immunotherapy has been recognized as a new and promising approach for various tumors. EC cells present a high tumor mutation burden and harbor abundant tumor antigens, including tumor-associated antigens and tumor-specific antigens. The latter, also referred to as neoantigens, are immunogenic mutated peptides presented by major histocompatibility complex class I molecules. While current genomics and bioinformatics technologies have greatly facilitated the identification of tumor neoantigens, identifying individual neoantigens systematically for successful therapies remains a challenging problem. Owing to the initiation of strong, specific tumor-killing cytotoxic T cell responses, neoantigens are emerging as promising targets to develop personalized treatment and have triggered the development of cancer vaccines, adoptive T cell therapies, and combination therapies. This review aims to give a current understanding of the clinical application of neoantigens in EC and provide direction for future investigation.

Keywords: esophageal cancer, immunotherapy, neoantigen, cancer vaccine, adoptive cell therapy

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INTRODUCTION

EC ranks as the seventh most common malignant tumor and the sixth leading cause of cancer-related death worldwide. An estimated 572,000 new cancers of EC and 509,000 deaths occurred in 2018 (1). To date, multidisciplinary therapy involving neoadjuvant chemoradiotherapy followed by surgery has formed the standard treatment for local advanced EC (2, 3). However, only 29% of patients who undergo resection after chemoradiotherapy show a pathological complete response, and the incidence of grade 3 or 4 treatment-related adverse events during chemoradiotherapy are common (2). The long-term survival for patients with locally advanced EC remains unsatisfactory despite therapeutic improvements (4).

Immunotherapy, including immune checkpoint blockade (5, 6), cancer vaccination (7), and adoptive T cell therapy (8), has been explored as a novel strategy for improving survival outcomes of EC patients recently. Immune checkpoints on the surface of T cells are receptors that are crucial for preventing autoimmune damage to healthy tissues. However, tumor cells exploit immune

checkpoint inhibition to evade the immune response (9, 10). Immune checkpoint blockade therapy has dramatically changed the treatment of melanoma and advanced non-small-cell lung cancer (11, 12). Several randomized clinical trials have shown that immune checkpoint blockade is a promising prospect for the treatment of EC (5, 13, 14).

It is known that accumulation of genetic alterations can result in cancer development. Neoantigens, referred to as immunogenic mutated peptides in tumor cells, are presented by major histocompatibility complex class I molecules and stimulate a strong cytotoxic T cell–mediated immune response (15).

EC appears to be associated with a high tumor mutation burden and neoantigen load compared with esophageal adenocarcinoma (16, 17). Due to their strong tumor-specific immunogenicity, neoantigens are emerging as a promising target for tumor immunotherapy. Immune checkpoint blockade achieves tumor control by releasing immune inhibition, while neoantigens aim to elicit the immunogenicity of tumors and trigger cytotoxic T cell responses, thereby improving antitumor efficacy (18).

In this review, we focus on the clinical application of neoantigens and highlight advances in the use of neoantigens in cancer vaccines, adoptive T cell therapy, and combination therapy for EC, and we discuss the present barriers and strategies.

CANCER VACCINES

Traditional cancer vaccines mainly use tumor-associated antigens, which are also expressed in normal human tissue. Owing to their high tumor-specific and exceptional immunogenicity, tumor neoantigens are ideal targets for cancer vaccine design (19, 20). Neoantigen vaccines, a new type of tumor immunotherapy, can induce a strong, specific immune response and elicit stable therapeutic effects.

New York esophageal squamous cell carcinoma 1 (NY-ESO-1) is known as a member of the testis cancer gene family with high immunogenicity. Oshima et al. screened 1,969 patients with various cancers and identified serum NY-ESO-1 antibody as a tumor-specific biomarker for EC (21). Subsequently, Wada et al. performed a phase I clinical trial using NY-ESO-1 protein as a cancer vaccine for eight patients with advanced EC (22). Antibody, CD4+, and CD8+ T cell responses were recognized in 87.5% (7/8), 87.5% (7/8), and 75.0% (6/8) of patients, respectively. Nevertheless, disease progression was eventually observed in all patients after vaccination for various reasons, such as primary tumor growth or paratracheal or abdominal lymph node metastases.

In a phase I dose-escalation trial of a two-dose cohort, cholesteryl pullulan (CHP)-NY-ESO-1 vaccine was confirmed to be safe and to induce immunogenicity in patients with advanced metastatic EC. The 200 μg dose cohort elicited stronger immune responses and displayed better survival outcomes than the 100 μg dose cohort (23). In addition, a phase II comparative study of CHP-NY-ESO-1 vaccine was performed in NY-ESO-1-expressing patients with esophageal squamous cell carcinoma who underwent neoadjuvant chemotherapy followed by surgery. No significant difference in survival benefit was noted between the CHP-NY-ESO-1 vaccine group and untreated controls (24).

Several studies have shown that the neoantigen vaccine can successfully induce cytotoxic T cells and trigger immune effects (25–27). However, sufficient and persistent immune effects are difficult to achieve with antitumor vaccines (25, 26). Rosenberg et al. suggested tumor cells possibly possess multiple immune escape mechanisms, such as insufficient tumor antigen expression, the tumor produces local immunosuppressive factors, T cells are "tolerized", or there is downregulation of T cell receptor signal transduction, among other things (28).

Compared with traditional peptide vaccines, mRNA vaccines are a genetic vaccine that might stimulate an effective immune response. Notably, Forghanifard et al. were the first to report that a novel chimeric mRNA-loaded dendritic-cell (DC) vaccine can elicit an effective immune response and induce cytotoxicity against EC *ex vivo*. Although this was a preclinical study, it provided a new approach to use DC-based vaccines for EC immunotherapy (29).

ADOPTIVE T CELL THERAPY

Tumor-specific neoantigens can also serve as promising targets for adoptive cell therapy. This involves patient T cells that have been genetically engineered to target a tumor cell surface antigen. The current strategies mostly use antigen-specific T cell receptor (TCR) gene-engineered T cells, chimeric antigen receptor (CAR) T cells, or tumor-infiltrating lymphocytes (TIL). These therapies have yielded remarkable tumor responses in clinical trials of hematological tumors and high mutation tumors (30, 31). However, these therapeutic options have limited therapeutic effect or are poorly understood in solid tumors.

Kageyama et al. conducted a first-in-man trial using adoptive cell therapy of MAGE-A4 T cell receptor gene-transduced lymphocytes in patients with MAGE-A4-expressing EC. Results revealed that T cell receptor gene-engineered T cells can be detected in the peripheral blood of all patients at the initial administered level for 1 month. Notably, transferred T cells in five patients persisted for more than 5 months. However, the antitumor effect was limited. Despite the long persistence of transferred T cells in blood, disease progression occurred in 70% (7/10) of patients after treatment. It is noteworthy that 30% (3/10) of patients with minimal tumor lesions had progression-free survival for 27 months (32). These findings suggest that adoptive T cell therapy may provide better survival outcomes in patients with early-stage tumors compared with those with advanced or recurrent EC. An explanation may be that various mechanisms underlie the benefits of adoptive T cell therapy. Another explanation might be cross-reactivity to corresponding wild-type peptide sequences in the identification of neoantigens. A recent study reported the establishment of a protocol for effective construction of neoantigen-specific TCR T cells to improve therapeutic outcomes (33).

Advances in genomics and bioinformatics have accelerated the emerging technology of CAR T cells, which transformed the field of adoptive T cell therapy. To our knowledge, no clinical trial using neoantigen target CAR T cell therapy in EC patients has been published. It has been reported that CAR T cells targeting EphA2 receptors stimulated antitumor activity of EC cells in a preclinical trial (34). The only clinical trial of CAR T cell therapy for EC, targeting the EpCAM adhesion molecule, is ongoing (NCT03013712). Both EphA2 and EpCAM are tumor-associated antigens that are overexpressed in patients with esophageal squamous cell carcinoma but are not tumor-specific antigens (34–36).

TIL is another candidate for T-cell therapy. Several clinical studies have demonstrated successful adoptive TIL therapy for solid tumors such as melanoma and ovarian cancer (37–40). In EC, TIL positivity is associated with better survival outcomes (41). Tan et al. used TIL to construct neoantigen-specific TCR T cells for esophageal squamous cell cancer and identified antitumor activity *in vivo* and *in vitro* (42). Due to this study only enrolling one patient, it is difficult to comprehensively assess the efficacy of adoptive TIL therapy. We recommend future studies enroll larger populations. To date, a clinical trial of adoptive TIL transfer therapy for EC has not been reported.

COMBINATION THERAPY

Although neoantigen-specific cancer vaccines or adoptive T cell therapy can elicit specific immune responses, tumor cells can escape from the immune system through multiple mechanisms (43–45). Additionally, the tumor microenvironment is rather complex and quite different from an *in vitro* milieu. Components derived from a neoantigen-enriched tumor microenvironment could suppress the function of effector T cells (46, 47). Therefore, combinations of neoantigen immunotherapy and different modes of treatment are now much more attractive.

Studies found that cancer vaccines could stimulate the proliferation of specific T cells; however, the expression of the programmed death ligand PD-L1 correspondingly increased (48, 49). This might suggest that cancer vaccines combined with a PD-L1 inhibitor or a PD-1 receptor inhibitor may be a possible therapeutic strategy. In a preclinical trial, Ishihara et al. tested a combination therapy of CHP-NY-ESO-1 cancer vaccine and an anti-PD-1 monoclonal antibody and induced significant tumor suppression ($P = 0.029$) compared with the no-treatment group (27). This combination therapy is a promising strategy and merits future attention.

Beyond this, it has been reported that a tumor-specific vaccine combined with immune checkpoint therapy can lead to a more efficient response than monotherapy in pancreatic and prostate cancer in preclinical comparative studies (50, 51). A neoantigen-specific cancer vaccine combined with adoptive T cell therapy has also been successfully used to achieve an antitumor response. Kageyama et al. conducted a phase I clinical trial of MAGE-A4 peptide vaccinations combined with TCR gene-engineered T cell in EC. Persistence of tumor-specific reactivity was detected although tumor regression was not observed.

Chemotherapy and radiation are important components in the treatment arsenal for EC patients. Several studies have confirmed that chemotherapy or radiotherapy can stimulate tumors to release antigens and induce the production of tumor antigen-specific effector cells in tumor tissue (52, 53). In EC, a phase I clinical trial of a multiple-epitope peptide vaccine combined with chemoradiation therapy has successfully been performed. Notably, six of 11 patients achieved complete response, and four patients with complete response experienced long-term survival benefit (54). Approaches to integrate chemoradiotherapy with immunotherapy provide novel therapeutic strategies and are now in the ascendance.

SUMMARY AND FUTURE DIRECTIONS

This review summarized the trials with neoantigens for EC or solid tumors including EC (**Table 1**) and the major types of neoantigens in EC with clinical application (**Figure 1**). Compared with immunotherapy based on tumor-related antigens, neoantigen-specific cancer vaccines or adoptive T cell transfer can drive specific immune responses and reduce adverse effects on normal tissue. However, effective antitumor activity and even survival benefit are currently not readily achievable with neoantigen monotherapy (55, 56). The maturation of technologies identifying non-synonymous mutations and evaluating the immunogenicity of mutated peptides will determine the success of future clinical applications (15, 57). Although substantial challenges lie ahead on the road to enabling neoantigen-specific effector T cells to completely eliminate tumor cells, recent advances in whole exome sequencing and bioinformatics technology will greatly facilitate the journey (58–60). There are three major strategies for the selection of candidate neoantigens—*in silico* peptide prediction, mass spectrometry-based immunopeptidomics, and whole-exome sequencing database list (15).

Better understanding the mechanisms of resistance to immunotherapy may enable the development of new strategies to improve clinical outcomes. While tumor cell intrinsic factors for resistance of immunotherapy remain poorly understood, published evidence points to the possibility of alterations in interferon (IFN)-gamma signaling pathways and absence of antigen presentation (61). It is already documented that IFN-gamma is the primary cytokine involved in the recognition and elimination of mutant cells in the classical signaling pathway (62). However, tumor cells could escape this effect by upregulating inhibitory ligands that impede the response of T cells or by inducing mutations in the IFN-gamma signaling pathway, resulting in immune evasion (63, 64). In addition, neoantigens or immunogenic mutated peptides are processed and presented on human leukocyte antigen (HLA) molecules of cancer cells. This observation raises the possibility that resistance to tumor-infiltrating lymphocyte adoptive cell transfer therapy could be mediated through silenced HLA molecules (65).

Tumor cell extrinsic factors may also contribute to resistance. In addition to tumor cells and immune cells, as well as the surrounding stroma, abundant immunosuppressive cells, including T regulatory cells, myeloid-derived suppressor cells, and tumor-associated macrophages, in the tumor microenvironment have important

TABLE 1 | List of trials with neoantigens for EC or solid tumors including EC.

	Target	Sponsor	Phase	Sample size	Trial identification	Outcome summary
Peptide vaccine	CHP-NY-ESO-1	ImmunoFrontier, Inc.	I	25	NCT01003808	The safety and immunogenicity were confirmed
	CHP-NY-ESO-1	Japan Agency for Medical Research and Development	II	54	UMIN000007905	DFS in 2 years: 56 *vs.* 58% in the vaccine arm and control arm; OS in 2 years: 76 *vs.* 79%, respectively
	HLA-A*2402	Shionogi & Co., Ltd	I	15	UMIN000023324	Cytotoxic T cell response was induced in all patients
Adoptive T cell therapy	MAGE-A4	Mie University	I	10	UMIN000002395	3 patients remained free from PD, survived for more than 27 months
	MAGE-A4	Tianjin Medical University Cancer Institute and Hospital	I	15	NCT01694472	N/A
	MAGE-A4	Adaptimmune	I	52	NCT03132922	Ongoing
	NY-ESO-1	Shenzhen Second People's Hospital	I	36	NCT02457650	N/A
	NY-ESO-1	University Health Network, Toronto	I	22	NCT02869217	Ongoing
Combination therapy	CHP-NY-ESO-1	ImmunoFrontier Inc.	I	26	UMIN000008006	8 patients had SD
	HLA-A*2402	Teikyo University	I	11	NCT00632333	6 patients of CR and 5 patients of PD were observed

EC, esophageal cancer; CHP, cholesteryl pullulan; NY-ESO-1, New York esophageal squamous cell carcinoma 1; MAGE-A4, melanoma-associated antigen A4; HLA, human lymphocyte antigen; DFS, disease-free survival; OS, overall survival; CR, complete response; SD, stable disease; PD, progressive disease; N/A, not available.

roles in the activity of cytotoxic T cells (66, 67). Spranger et al. showed that tumor immune escape is associated with a mechanism whereby a cancer vaccine could elicit T cell activation and upregulate regulatory T cells simultaneously (68). Moreover, Eil et al. found that tumor necrosis releases intracellular potassium ions and blocks the ionic checkpoint, resulting in inhibition of the effector T cell response (69). Hence, the process of tumor immunogenicity and immune escape involves multiple mechanisms, and there remains much to explore in future research.

To overcome the resistance of immunotherapy, contemporary approaches primarily focus on the combination therapy of neoantigen-specific immunotherapy and traditional therapy. As described previously, chemotherapy or radiotherapy might facilitate effector T cell infiltration by eliciting tumor tissue to

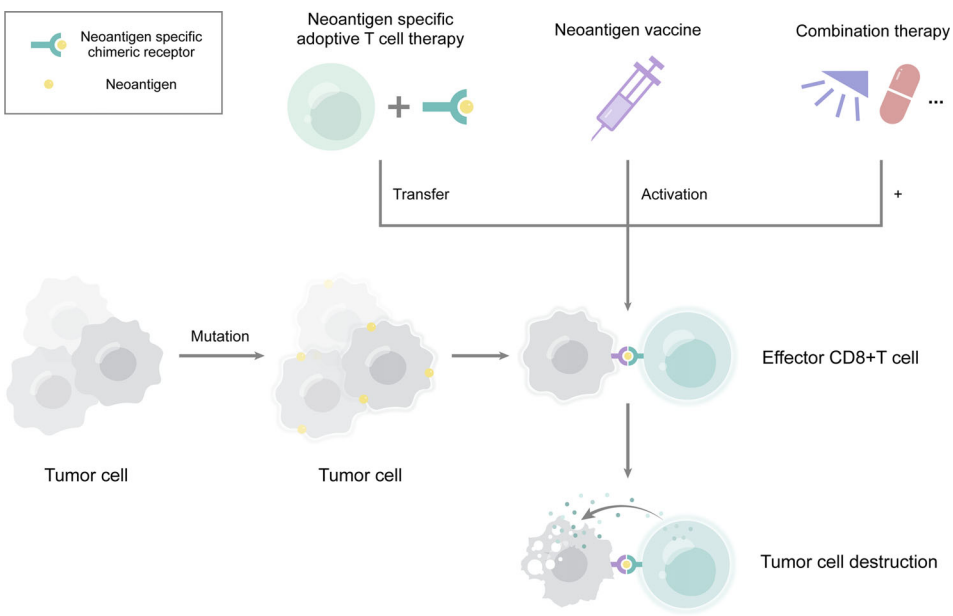

FIGURE 1 | The major clinical application of neoantigens in esophageal cancer. Neoantigens derived from mutations in tumor cells. They can serve many purposes, including neoantigen vaccination, neoantigen specific adoptive T cell therapy, and combination therapy. Combinations of neoantigens with radiotherapy, chemotherapy, or immune checkpoint inhibitor therapy may lead to a better effect. Neoantigens induce the production of effector CD8+ T cells, thereby eliminating tumor cells.

release more antigens (52, 54). Another actionable approach involves the use of neoantigen-specific immunotherapy and PD-1 or PD-L1 inhibitor therapy. The rationale for this combination is that blocking active checkpoints might reactivate T cell function (70). Furthermore, the strategy combining molecularly targeted therapy with immunotherapy has been studied in patients with melanoma, and a synergistic effect has been observed (71). These combination strategies may be the potential future directions in the treatment of EC.

As an emerging therapeutic approach, neoantigen-specific immunotherapy has shown potential therapeutic effect on EC in several preclinical and clinical trials. Here, we provide a clear picture of the clinical application of neoantigens in EC from tumor-specific cancer vaccines and adoptive T cells to combination therapy. We also discuss the current barriers and strategies, thus providing direction for future investigation.

AUTHOR CONTRIBUTIONS

L-QC and YY conceptualized the study, revised the manuscript, and supervised the study. Y-MG and YZ conceptualized the study, drafted the manuscript, and made the figures. Y-MG and YZ collected the literature and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Promise of Personalized TCR-Based Cellular Immunotherapy for Cancer Patients

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Mutation-derived neoantigens are now established as attractive targets for cancer immunotherapy. The field of adoptive T cell transfer (ACT) therapy was significantly reshaped by tumor neoantigens and is now moving towards the genetic engineering of T cells with neoantigen-specific T cell receptors (TCRs). Yet, the identification of neoantigen-reactive TCRs remains challenging and the process needs to be adapted to clinical timelines. In addition, the state of recipient T cells for TCR transduction is critical and can affect TCR-ACT efficacy. Here we provide an overview of the main strategies for TCR-engineering, describe the selection and expansion of optimal carrier cells for TCR-ACT and discuss the next-generation methods for rapid identification of relevant TCR candidates for gene transfer therapy.

Keywords: cancer immunotherapy, T cell receptor, gene transfer therapy, adoptive cell transfer, cell engineering, T cells

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INTRODUCTION

Pioneered by Rosenberg and colleagues, adoptive cell transfer (ACT) therapy is an immunotherapy strategy relying on the infusion of autologous tumor-infiltrating lymphocytes (TILs) to cancer patients. ACT demonstrated promising clinic outcomes in melanoma; with durable responses in 10-20% of patients (1, 2). Despite this progress, however, the majority of patients does not respond (3, 4) and the efficacy of ACT remains limited to melanoma and cervical cancer (5). One of the possible reasons for this limitation is the low frequency of antigen-specific T cells in TILs (1–3, 6–8). Furthermore, the proportion of bystander (i.e. tumor unrelated) TILs, such as viral-specific T cells, can be quite high in cellular products (9). Also, it was recently demonstrated that current cell culture conditions do not lead to a consistent clonal expansion of *ex vivo* TILs but rather lead to a biased immune repertoire (10). Altogether, these observations brought on the hypothesis that the proliferative potential of tumor antigen-reactive TILs is likely to be limited, therefore leading to their relative dilution *in vitro* by overgrowing bystander TILs.

To circumvent these issues, the field is moving towards the genetic engineering of T cells to express either chimeric antigen receptors (CARs) (11) or tumor antigen-specific T cell receptors (TCRs) (**Figure 1**) (12–17). CD19-targeting CAR T cells mediated complete responses in about 80% of patients with B cell acute lymphoblastic leukemia cancer (18). Yet, CARs target tissue-restricted antigens expressed on the surface of tumor cells (19), thus limiting their applications (20), while TCR-transduced

cells can target any surface or intracellular antigen. In this report, we will focus on TCR-based cellular immunotherapy (**Figure 1**).

TCRs are heterodimers consisting of disulfide-linked α and β chains, each with a variable and a constant domain (21, 22). The variable regions can bind the antigen-MHC complex. The binding domain is constructed based on the recombination of multiple gene segments, leading to the great diversity of the TCR repertoire, with potentially $>10^{15}$ distinct αβ TCRs (23–25). TCR gene-transfer therapy targeting tumor-associated antigens (TAAs), such as MART-1 and NY-ESO-1, achieved clinical responses ranging from about 10 to 60% of patients from different malignancies (12, 14, 26, 27). Despite these promising clinical outcomes, efficacy remained limited and important toxicities occurred (13, 27, 28). The field was then rejuvenated by the perspective of using TCRs targeting private neoantigens, which have emerged as clinically relevant targets (29, 30). In this review, we will discuss several issues including the common tools used for cells transduction, the optimal cells to transduce for ACT and the acceleration of the identification of relevant tumor-specific TCRs for gene transfer therapy.

STRATEGIES FOR TCR-ENGINEERING OF T CELLS

Viral Vectors

As for CAR-T cell based therapy, viral vectors were widely exploited for *ex vivo* TCR gene transfer into recipient T cells (**Figure 2**). In clinical trials, the most common viral systems used are gamma retrovirus- (RV) and lentivirus-based vectors (LV), as reviewed in (31). Both RVs and LVs allow for stable integration and efficient long-term expression of exogenous TCRs. However, safety concerns to RVs and LVs remained, that are mainly insertional mutagenesis and neoplastic transformation (32), as well as generation of replication-competent viral particles. The latter limitations boosted the development of novel vector designs, such as addition of insulator sequences (33), disruption of the long terminal repeats for self-inactivating viral vectors (34, 35) or pseudotyping (36). Of note, RV transduction requires mitotic cells for the transgene to penetrate the nucleus and integrate in the genome and thus recipient cells must be activated beforehand. Conversely, LVs allow the effective transduction of a variety of not actively dividing and terminally differentiated cells. Yet, human resting T cells are scarcely susceptible to transduction by LVs and need to be minimally stimulated to enter the G1b phase (37, 38). Importantly. several studies highlighted a positive correlation between the proliferative potential of adoptively transferred cells and their *in vivo* persistency (1), sustaining the use of LVs in the clinic to limit recipient cell stimulation *in vitro*.

As alternative to RVs and LVs, adeno-associated virus-derived vectors (AAVs) can be used for TCR-engineering. AAVs are replication-deficient systems and are not known to induce any side effects, making them ideal candidates from a safety standpoint. AAV-based cancer treatments have not yet been used in the clinic, however multiple strategies have been developed and hold great promise for the future (39–41). It is to

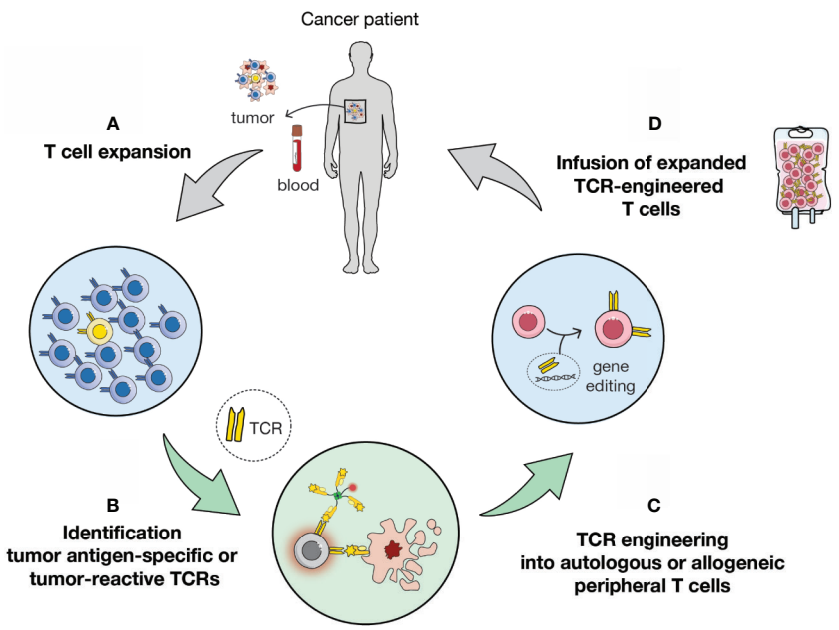

FIGURE 1 | Overview of TCR-based immunotherapy. **(A)** Expansion of T cells from tumor or blood samples of patient or healthy donors. **(B)** Identification of antigen-specific or tumor-reactive T cells and of cognate T cell receptors (TCRs). **(C)** Transduction of autologous or allogenic carrier cells. **(D)** Adoptive cell transfer of TCR-engineered cells to the patient.

be noted that vector manufacturing remains time consuming (over six months) and expensive (42).

Non-Viral Systems

Non-viral methods for stable gene transfer into T cells were investigated in CAR-T clinical trials and represent a future opportunity for TCR gene transfer clinical studies. Non-viral engineering strategies include transposon-based vector systems, such as "Sleeping Beauty", by which a transgene of interest flanked by inverted terminal repeats is provided to target cells as discrete DNA molecule and randomly integrates in its genome when a transposase is co-supplemented *in trans* (i.e. mRNA, plasmid or protein) (43). Over the past decades, transposons and transposases have been extensively optimized to increase their activity and reduce toxicity (e.g. insertional mutagenesis) (44). Alternatively, exogenous TCRs can be transiently expressed *in trans* if transfected into T cells in the form of mRNA molecules or non-integrating vectors (45, 46). All these non-viral systems may be preferable to viral ones for the clinic because of their easier handling and cheaper production costs. Of important note, gene editing of T cells with TCRs, as opposed to CARs, has to face an extra challenge. Mispairing of exogenous and endogenous TCR chains can indeed occur and lead to off-target toxicity. Researchers have therefore developed several platforms to specifically silence the expression of the endogenous TCR, based on Sleeping Beauty, Zinc finger nucleases (47), transcription activator-like effector nucleases (TALEN) (48, 49), mega-nucleases or Clustered Regularly-Interspaced Short Palindromic Repeats (CRISPR)–Cas9 (50, 51) (**Figure 2**).

The CRISPR-Cas9 technology relies on short RNA sequences which are used to target the site of insertion instead of proteins and which are easily synthesized *in vitro*. As such, CRISPR-Cas9 enables the simultaneous targeting of multiple genome sites and site-specific mutagenesis, allowing the knockout (KO) of endogenous TCRs or of immune checkpoint such as PD-1 (52) and the induction of cytokine expression (e.g. IL-7, IL-12, IL-15, IL-18) (17) (**Figure 2**). The safety and feasibility of multiplex CRISPR-Cas9 gene editing of T cells was demonstrated in a study with advanced refractory cancer patients infused with autologous T cells KO for the three genes *TRAC*, *TRBC* and *PDC1*

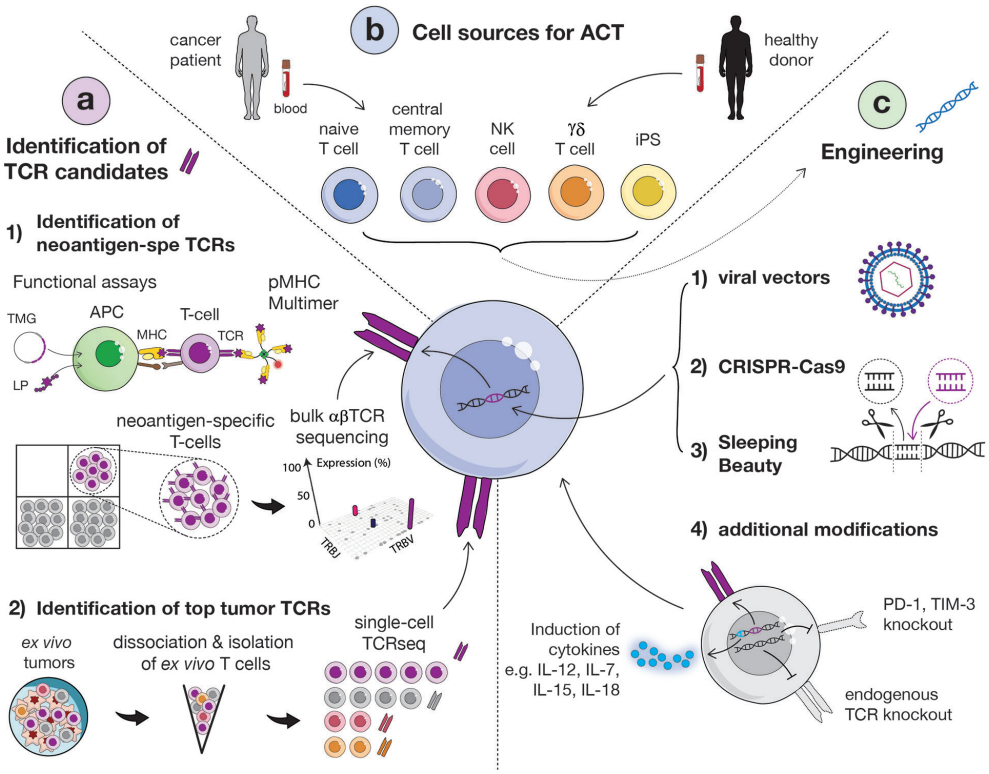

FIGURE 2 | Overview of main strategies to identify relevant TCRs, methods for transduction and options of carrier cells. **(A)** Identification of TCR candidates *via* functional assays and sequential isolation of neoantigen-specific T cells based on pMHC multimer staining. Isolated neoantigen-reactive T cells can then be sequenced in bulk for the identification of dominant TCR clonotypes (1). Alternatively, tumor-specific TCRs can be identified directly from *ex vivo* fresh tumor, without intermediate culture, by sorting T cells by flow cytometry and performing single-cell TCR sequencing (2). (APC, antigen presenting cell; MHC, major histocompatibility complex; LP, long peptide; TMG, tandem minigene). **(B)** Several carrier cells can be used for TCR transduction, including αβ (CD4 or CD8) T cells, naïve or central memory cells, NK cells, γδ T cells and induced-pluripotent stem cells (iPSCs). Carrier cells can be derived from healthy donor or patient blood. **(C)** TCR engineering into carrier cells can be obtained *via* viral (1) or non-viral vectors [such as CRISPR-Cas9 (2) or Sleeping Beauty (3)]. Additional modifications of cells to enhance their functionality and *in vivo* persistency but also avoid TCR mispairing can be performed, such as the knock-out of endogenous TCR or immune inhibitor genes and the induction of cytokines (4).

(PD-1 loci) and transduced with a NY-ESO1 TCR (53). Of interest, the KO by CRISPR-Cas9 of cytokine-induced SH2 protein (CISH), an immune checkpoint, was found to increase the *in vitro* proliferation and functionality of TCR-engineered T cells and such strategy will be further explored in an upcoming clinical trial (NCT04426669) (54). Despite technical challenges which still need to be overcome, in particular regarding the delivery systems currently based on AAV and transfection (55–59), CRISPR-Cas9 will likely become the method of choice for therapeutic gene engineering in the upcoming years.

SELECTION AND EXPANSION OF OPTIMAL CELLS FOR ACT

T Cells

T lymphocytes are the most common source of cells used as carrier for gene transfer therapy. Following TCR engineering into recipient cells, TCR-T cells need to be expanded to reach sufficient numbers for ACT. The primary starting material is most often autologous peripheral blood (**Figure 2**).

Thus far, TCR gene therapy mainly focused on CD8 T cells, which represent key players of ACT. In particular, a retrospective analysis of TIL ACT infusion products and clinical efficacy from 92 patients highlighted an association between the fraction of CD8 T cells and clinical benefit (60). There is, however, an emerging clinical relevance of CD4 T cells. Early evidence was provided by Tran and colleagues who showed the antitumor potential of neoantigen-specific CD4 TILs by ACT (61). Of interest, the direct cytolytic capacity of CD4 T cells was demonstrated (62–64). In addition, CD4 T cell's help is essential to generate efficient tumor-reactive effector CD8 T cells (65), notably during the process of epitope spreading (29, 66). Yet TCR gene transfer therapy with CD4 tumor-specific TCRs is also limited by the challenging prediction and detection of MHC class II-restricted neoantigen-specific CD4 T cells, despite major advances (67, 68).

T cell differentiation state is critical for ACT. The profile of adoptively-transferred cells is indeed likely to affect their *in vivo* persistency and thus treatment efficacy. T cell differentiation states range from naïve to central memory, effector memory and finally terminally differentiated (EMRA) (69). Accumulating evidence shows that the effector phenotype acquired *in vitro* negatively impacts the antitumor potential of T cells *in vivo* (70), while ACT efficacy requires a long-term persistence of transferred cells. Thus, less-differentiated T cell populations that maintain self-renewal capabilities are preferred and were associated with improved clinical benefit (71, 72). Naïve and memory T cells maintain the highest proliferative potential combined to the most potent fitness and stemness (73–76) (**Figure 2**). Consistently, naïve and memory subsets were found more effective than effector T cells for ACT (70, 74–77). Furthermore, Hinrich and colleagues have demonstrated that naïve CD8 T cells had a higher anti-tumor potential for ACT as compared to central memory cells (77).

The T cell state is also modulated during the expansion phase *in vitro*. TCR-transduced T cells are commonly expanded with anti-CD3/CD28 beads in the presence of IL-2 (12, 38, 78). It has been demonstrated that the addition to the culture medium of alternative cytokines, such as IL-7, IL-15 and/or IL-21, enabled to the generation of less-differentiated TCR-engineered T cells thus leading to increased persistency and ultimately improved efficacy (71, 79–81).

Autologous T cells are available in limited quantities and their state is likely to be affected by the multiple rounds of cancer treatments which patients undergo before ACT, complicating the manufacturing process and the feasibility of TCR gene transfer therapy. Therefore allogenic universal T cells have also been exploited for gene transfer therapy (82) (**Figure 2**). A few issues are to be noted, including competition with endogenous TCRs, mispairing of TCR subunits, risk of off-target toxicity due to allogenic TCR-T cell infusion and ACT product rejection by the host (83). It is thus required to KO endogenous TCRs to improve the safety and efficacy of TCR-ACT with allogenic cells (49). For all these reasons, several alternative non-T cell types were evaluated.

Alternative to Autologous αβ T Cells

Mensali and colleagues provided the first proof of concept that cells other than αβ T cells could be used as recipient cells for TCR gene therapy. They transferred a TCR into NK cell line, NK-92, and demonstrated efficacy *in vivo* (84). More recently, Parlar and coworkers have engineered NK cells with a tyrosinase-specific TCR and highlighted their cytolytic potential *in vitro* (85). A potential benefit of using NK cells as a carrier is their ability to remain cytotoxic in an MHC-independent manner. This could be of interest in case of MHC loss, which is a common immune suppressive mechanism exerted by tumors (86). Alternatively, γδ T cells can also be TCR-engineered (**Figure 2**), thus avoiding the issue of TCR mispairing. The latter strategy was proven efficient in leukemia (87). Furthermore, as T cell exhaustion is a critical component of ACT efficacy, there is an increasing interest in using induced-pluripotent stem cells (iPSCs) for gene transfer therapy (**Figure 2**). Nishimura and colleagues were able to generate iPSCs from antigen-specific CD8 T cells and to re-differentiate them. In this way, they obtained rejuvenated cells with longer telomeres and a high proliferative potential, making them fitter for therapy (88). The efficacy of TCR-engineered iPSCs was shown *in vivo* (89).

IDENTIFICATION OF RELEVANT TCRS FOR GENE TRANSFER THERAPY

As mentioned previously, neoantigens are attractive targets for TCR-based therapy. To date, most neoantigens originated from non-synonymous mutations. T cell reactivity to neoantigens was associated with improved clinical benefit of immunotherapy, both immune checkpoint blockade (90–92) and ACT (93–95). Early reports showed promising results in terms of safety, feasibility and

efficacy with neoantigen targeting immunotherapy either in the form of vaccination (29, 96–98) or ACT (30, 99). Strikingly, complete remissions were observed following infusion of neoantigen-reactive T cells, highlighting the potency of mutanome based therapies (30, 99).

The first key challenge lies in the identification of neoantigens, which is a long and tedious process and was reviewed elsewhere (100). Upon neoantigen identification, neoepitope-specific T cells are purified and their TCR is sequenced. Candidate TCRs are then cloned to validate their antigen specificity and tumor reactivity (**Figure 2**).

Neoantigen Identification

Neoantigen-specific T cells, and hence their cognate TCR, can be identified in different samples including tumor or blood from patient or naïve T cells from healthy donors. Briefly, neoantigens can be identified from expanded TILs (7, 95, 101). However, due to low frequencies, sensitive detection sometimes requires antigen-specific *in vitro* stimulation (IVS). Of interest, we developed a novel strategy to improve the detection of neoantigens in TILs, based on the addition of pools of predicted neo-epitopes at the initiation of the TIL culture (7). Alternatively, TILs can be enriched prior to culture by sorting of dissociated tumor material *ex vivo*, based on various activation markers like PD-1 (CD279), OX40, CD137 (4-1BB), CD39 and CD103 (102–106).

Peripheral blood lymphocytes (PBLs) can also be exploited for neoantigen identification. IVS with antigen presenting cells (APC) loaded with neoantigen candidates have been extensively used to detect neoantigen reactivity (7, 30, 96, 107–110). Prior enrichment strategies have also been used by different groups including: the isolation of memory (107) or naïve T cells (108) or the sorting with PD-1 (109). Most identification processes use PBLs from autologous origin but it has been shown to be possible from allogenic sources as well (108).

Neoantigen-Specific T Cell Isolation and TCR Repertoire Analysis

Upon their identification, neoantigen-specific T cells can be purified based on pMHC multimer staining or based on the up-regulation of activation markers following specific-activation, such as 4-1BB and OX40 (7, 107, 108, 111, 112). Isolated cells then undergo bulk α and β TCR sequencing in order to select the dominant α and β TCR clonotypes. Of note, TCR repertoire analysis strategies are challenging due to the high diversity of TCR repertoires (113). Advances in next-generation sequencing has improved the interpretation of TCR repertoires. Using RNA as a source of material allows allelic exclusion thereby avoiding to overestimate repertoires diversity. RNA is also more sensitive than DNA despite being less quantitative due to variation in expression levels (114). Among the different sequencing methods, multiplex polymerase chain reaction (PCR) (115) remains the most commonly used strategy, despite misrepresentation of clonotypes proportion introduced by heterogeneity in primers efficiency (116). Other approaches rely on the addition of adaptors prior to PCR amplification (117, 118), such as the 5' RACE PCR or TCR amplification following gene capture (119). For each method, the bias in quantification reduces the ability for easy pairing of α and β chains, which is required for therapeutic applications. A concept based on multiple sequencing and combinatorial analysis was developed to pair αβ TCR chains, yet this strategy is limited to high-frequency clonotypes and requires large cell numbers (120). To avoid the above-mentioned limitations, both T cell cloning or single-cell sequencing can be used.

Antigen Specificity Validation of Candidate TCR Pairs

Interrogation of neoantigen-specificity and antitumor-reactivity of candidate TCRs can be assessed following the expression of TCR candidates into recipient cells, a strategy hereafter referred as TCR cloning. Antigen-specificity can be challenged by transducing TCRs into activated PBLs or Jurkat T cells which are then co-cultured with APCs loaded with neoantigens (29, 86, 94, 95, 99, 101, 102, 107, 121–123). Next, tumor-reactivity can be measured by co-culture of TCR-engineered T cells with autologous tumor cells or APCs pulsed with tumor lysate (86, 94, 101, 102, 121, 124, 125). To enable a rapid identification of neoantigen-reactive TCRs, Paria and colleagues have developed a TCR cloning methodology using Jurkat T cells electroporated with RNAs encoding TCR α and β chain, respectively (126). The benefit of this approach lies in the use of RNA electroporation, which is faster and more efficient than TCR transduction by genetic engineering. TCR expression is transient but sufficient for TCR interrogation. Interestingly, they used the Jurkat luciferase system (under NFAT promotor) which is a rapid and easy read-out.

Of important note, the reactivity of validated neoantigen-specific TCRs to the wild-type peptide should be evaluated to avoid autoimmunity and thus ensure patient safety. This can done by performing a peptide dose response with APCs (86, 94, 95, 99, 102, 107). Another method consists of using a high-throughput genetic platform (127).

Selection of Tumor-Specific TCRs By Single-Cell Technologies

As an alternative to the aforementioned time-consuming strategies (based on TCR isolation and downstream TCR validation), new developments enable the direct identification of tumor-specific TCRs by pre-selecting the most frequent clonotypes from fresh tumor samples (121) (**Figure 2**). Selected αβ TCR pairs are then challenged against tumor cells by TCR cloning. Others have however found that the tumor reactivity of intra-tumoral TCR repertoire was low (125), highlighting a potential limitation of using this strategy for gene transfer therapy. Identified tumor-reactive TCRs can then be examined retrospectively for antigen specificity using ligand discovery approaches, based for instance on trogocytosis, a process in which T cells exchange membrane proteins with APC presenting candidate antigens (128). Other possibilities for TCR ligand discovery include the use of chimeric signaling and antigen presenting bifunctional receptors (SABRs) (129).

It is to be noted that the development of microfluidic-based platforms will accelerate and automate the isolation of relevant TCRs for TCR-ACT in the near future (130–132).

Instead of direct TCR identification, combined single-cell transcriptomics and TCR sequencing (133) could be exploited to define intra-tumoral signatures of neoantigen-specific or tumor-reactive TCRs (134). Old-generation single-cell methodologies are tedious and allow the sequencing of a limited number of cells (135, 136). Single-cell TCR sequencing and profiling of T cells was recently eased by the development of a commercially available strategy by 10x Genomics (137–139). Briefly, the latter microfluidic-based technology is able to generate an emulsion containing one trapped cell with a uniquely barcoded bead and, upon cell lysis polyadenylated mRNA is captured. The process results in barcoded libraries, which undergo both downstream single-cell TCR sequencing and transcriptomic profiling. Overall, 15'000-20'000 cells can be covered. The 10x Genomics strategy allows the analysis of clones in a timely manner and was applied for the identification of a signature of clone persistency in the circulation after ACT (140). In the near future, intra-tumoral signatures of neoantigen and/or tumor-reactive TCRs may be defined based on a comprehensive database of TCRs combined to their *ex vivo* transcriptomic profiling. We foresee that these signatures will enable the direct identification of relevant TCR pairs from *ex vivo* tumor, which will considerably facilitate and accelerate the selection of TCR candidates for gene transfer therapy.

DISCUSSION AND FUTURE PERSPECTIVES

In this review, we provided an overview of the available therapeutic engineering systems, but also of the optimal carrier cells for TCR gene transfer therapy, and finally we described strategies to identify new TCR candidates. The field is constantly evolving and future advances are likely to reshape the landscape of TCR-ACT. Multiple clinical trials are currently ongoing in different types of cancer (17) and the results are awaited with much anticipation. In the future, we could take advantage of unique intra-tumoral transcriptomic signatures from identified neoantigen-specific or tumor-reactive TCRs. These signatures may then be used to directly and rapidly fish out TCRs of interest for gene transfer therapy. Challenges of TCR-ACT remain, in particular immune-editing following therapy, ultimately leading to immune evasion and progression (86). To increase the efficacy of TCR-ACT therapy and counter potential immune-editing, multiple TCRs should be targeted, which is currently possible with the CRISPR-Cas9 technology. Future strategies may also focus on the simultaneous targeting of MHC-restricted epitopes together with membrane antigens. Importantly, to extend the reach of therapy and benefit more cancer patients, 'shared' neoantigens (141, 142) arising from 'hotspots' mutations shared between unrelated individuals should be preferentially targeted and these approaches will be further explored in upcoming clinical trials (e.g. NCT03190941). Additionally, it is likely that next-generation improvement of carrier cells will further potentiate TCR-ACT efficacy. Given the plethora of existing technologies and their constant amelioration, together with the many ongoing clinical trials, we expect the immune-oncology field to be fundamentally modified by TCR gene transfer therapy in the upcoming years.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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REVIEW
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The Role of Neoantigens in Cancer Immunotherapy

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Somatic mutation-derived neoantigens, expressed only on tumor cells, may elicit antitumor T-cell responses in cancer immunotherapies with minimal immune tolerance. Neoantigens can be identified by multiple bioinformatics technologies, mainly based on whole-exome sequencing. Personalized cancer vaccines and adoptive T cell therapies are two primary treatment modalities targeting neoantigens, and both of them have shown promising therapeutic effects. This review, summarizes the history of neoantigen-related tumor control, introduces recent neoantigen screening and identification methods, and discusses the role of neoantigen in cancer immunotherapies. Moreover, we propose the challenges of targeting neoantigens for cancer treatment.

Keywords: neoantigen, immunotherapy, vaccine, adoptive cell therapy, personalized treatment

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INTRODUCTION

Immunotherapy has revolutionized the management of cancer treatment. Targeting the immune inhibitory regulators PD-1, PD-L1 or CTLA-4 is a common way to promote antitumor immune responses. However, immunotherapy boosting the immune system to destroy cancer cells has shown durable clinical responses in patients with various malignancies, but only in a subset of patients (1). Moreover, the non-antigen-specific simulation can activate global T cells and damage the human body, leading to immune-related adverse events, even fatal events (2). Thus substantial efforts are needed to explore more specific and powerful immunotherapy approaches, either alone or in combination.

As a genetic disease, cancer results from the accumulation of DNA damage and genetic alterations (3). These non-synonymous somatic mutations generate neoepitopes, which can be recognized by endogenous T cells as non-self-proteins and induce an antitumor immune response (1, 4). In most cases, neoantigens arise from single nucleotide mutations (SNV), gene fusion, alternative splicing, intron retention, insertion, and deletions. Other sources of neoantigens including post-translational modifications and endogenous retrovirus-associated tumors (5). The presentation of neoantigens is tumor-specific and able to elicit T cell-mediated antitumor immunity (4). Therefore, neoantigen is an ideal immunotherapy candidate. Two primary neoantigen-based therapeutic modalities have been explored in clinical practices: personalized vaccines and adoptive cell therapy (ACT) (6). Here we review the history of neoantigens about tumor control and

introduce recent neoantigen screening and identification methods. We also discuss the role of neoantigen in cancer immunotherapies and its current challenges.

HISTORICAL OVERVIEW OF TUMOR NEOANTIGENS

The foundation of cancer immunology can date back to the middle of the twentieth. Gross (7) reported that transplantable tumors could produce active immunity against syngeneic mice. Another research showed that carcinogen-induced tumors had antigenic properties that the immune system could identify (8). In 1988, De Plaen et al. (9) discovered the first neoantigen recognized by cytolytic T lymphocytes (CTLs) in a methylcholanthrene (MCA)-induced mouse tumor model. The recognized neoantigen also could be identified in a cDNA library. Then, CD4+ T cells stimulated tumor-specific immunity and inhibited tumor growth after recognizing neoantigens in mice (10). In 1996, mutated neoantigens recognized by tumor-infiltrating lymphocytes (TILs) and CTLs were found in human melanoma (11) and renal cell carcinoma (12), respectively. Several years later, Rosenberg and his colleagues (13) revealed nearly complete regression in a melanoma patient after receiving autologous tumor-reactive TILs. T cells reactivity against neoantigen was proved to predominate cellular antitumor response in a long-term surviving melanoma patient (14). A detailed analysis in a melanoma patient with complete tumor regression after adoptive TILs transfer indicated that tumor-reactive T cells could be persistent and relevant for tumor regression (15). Collectively, neoantigen has the potential to be the target of antitumor immunity.

Advances in next-generation sequencing (NGS) technologies have provided access to compare mutations in the normal and tumor genome in 2008 (16). Then NGS was used to demonstrate tumor-specific antigens in an immunogenic mouse model, which opened a new dimension for antigenic targets of cancer immunity (17). By using NGS, Castle et al. (18) assessed the antitumoral activity of the neoantigen vaccine in the B16 melanoma tumor model. Consequently, the vaccine-elicited T-cell immunogenicity showed protective effects, providing new insights into immunogenic neoantigen-based vaccine treatment in human cancers (18). NGS also contributed to identify neoantigens recognized by TILs in melanoma patients (19, 20) and suggested the feasibility of neoantigen-specific T cell reactivity analysis in human cancer immunotherapy (20). Neoantigen was identified as the target of checkpoint blockade immunotherapies in a sarcomas mouse model (21). Concurrently, the adoptive transfer of neoantigen-specific CD4+ T cells achieved tumor regression in a metastatic epithelial cancer (22).

Further investigations found that neoantigen burden was associated with immune checkpoint inhibitors' therapeutic responses in melanoma (23) and lung cancer patients (24). In 2015, Beatriz's team first reported that vaccination with neoantigen could augment T cell immunity in patients with advanced melanoma, demonstrating the efficacy and feasibility of a personalized dendritic cell (DC)vaccine (25). A subsequent study revealed that ACT targeting mutant KRAS neoantigen mediated antitumor immune system, leading to regression in patients with metastatic colon cancer (26). Furthermore, the correlation between clonal neoantigen burden and response to immune checkpoint blockade was defined, rendering it is possible to target clonal neoantigens in T cell therapies (27). Ott et al. treated melanoma patients with a peptide vaccine targeting 20 predicted personal tumor neoantigens (28). Consistent with the reactive T cells responses, four patients had no recurrent diseases, and two patients had complete tumor regression after anti-PD-1 was administrated to recurrence (28). Then individualized RNA mutanome vaccine also induced T cell infiltration and killed autologous tumor cells, resulting in tumor regression and durable survival (29). The personalized neoantigen vaccine and nivolumab combination proved safe and immunogenicity in advanced solid tumors (30).

IDENTIFICATION OF TUMOR-SPECIFIC NEOANTIGENS

With the advances of NGS, it has become feasible to acquire genomic changes in some kinds of tumors, which provided the basis of neoantigen identification (31). Thus, the current process of predicting candidate neoantigens mainly includes three parts: 1) identify tumor-specific mutations using whole-genome sequencing (WGS), whole-exome sequencing (WES), or RNA-seq; 2) predict major histocompatibility complex (MHC) types, which in humans are called human leukocyte antigen (HLA) and neoantigen presentation; 3) prioritize and select candidate neoantigens (4, 32).

WES should be performed to map the cancer mutanome from the tumor and paired normal tissue. Combining RNA-seq can determine whether the mutation is expressed in tumors and infer its relative frequency overlapped with exome-based variants (4, 33). The minimum requirements for immunogenic and potential therapeutic mutations include: 1) the mutant peptide must be processed and presented by MHC molecules, and 2) the presented peptide-MHC complex must be recognized by endogenous T cells (31). Antigen processing and presentation are complex but different processes for MHC class I and II molecules (34). Peptides binding to MHC-I molecules are usually of a small length of 8-10 residues, while MHC-II molecules could bind to longer peptides with a length of 11-20 amino acids (34–36). Several tools depend on NGS data from WES, WGS, or RNA-seq can be used to predict HLA alleles, including Optitype (37) and Polysolver (38) for class I alleles, seq2HLA (39), Athlates (40), HLAScan (41), HLAProfiler (42), PHLAT (43), and ArcasHLA (44) for both class I and II HLA alleles. Following this, computational algorithms [for example, NetMHC (45), NetMHCpan (46, 47), and MHCflurry (48)] have been developed to predict MHC binding affinity. The criteria of binding affinities (IC50) are defined as high, intermediate to weak, and non-binder with IC50 <150, 150-500, or >500 nM,

respectively (the commonly described binding affinity threshold) (49). The prioritization of candidate neoantigens can be achieved by the predicted binding affinities alone or in combination with the mutant expression level (50). On the other hand, mass spectrometry (MS) based immunopeptidome has enabled the discovery of thousands of MHC-associated neoantigens (51), and the combination of MS and WES is a powerful weapon to predict immunogenic tumor mutations (52). Compared to MS, T cell-based assays can directly detect whether an MHC-presented neoantigen has been recognized by T cells (4, 32). A variety of methodologies [e.g., fluorescently labeled HLA tetramers or multimers (53), the enzyme-linked immunosorbent spot (ELISpot) (54)] that stimulate and test neoantigen-reactive T cells have been reported. However, these cell-based assays are currently costly, time-consuming, and technically challenging (4).

CLINICAL APPLICATION OF NEOANTIGENS

The predicted tumor-specific neoantigens are relevant targets for clinical personalized immunotherapies, either as a vaccine or a cellular therapy product.

Vaccines

Personalized vaccines can be formulated as synthetic long peptide (SLP), DNA, RNA, DC, and viral and bacteria (55). Results from several clinical trials using neoantigen vaccines in patients with melanoma or glioblastoma are encouraging (25, 28, 29, 56, 57). Carreno et al. (25) first found that the DC vaccine augmented pre-existing neoantigen-specific immunity and induced previously undetected neoantigen induced T cell responses in three advanced melanoma patients. Neoantigens are processed and presented by HLA-A*02:01 molecules. Two subsequent clinical studies published in 2017 confirmed the potential of personalized neoantigen vaccine in treating patients with melanoma (28, 29). Ott et al. vaccinated six patients with SLP targeting up to 20 predicted tumor neoantigens (NCT01970358). Four of the six patients experienced no tumor recurrence in the following 25 months after vaccination, and another two patients with recurrence achieved complete tumor regression after received anti-PD-1 therapy (28).

Further analysis indicated that eight high-risk patients had durable neoantigens-induced T cell responses. It is encouraging that almost four years after being treated with vaccines, all are alive, and six have no active disease (58). Sahin et al. generated the first individualized RNA mutanome vaccines in 13 patients with stage melanoma (NCT02035956). Eight patients remained tumor-free within the follow-up period. Two of five patients with metastatic disease achieved objective responses, while one presented a complete response to RNA vaccine combined with PD-1 inhibitor (29).

Notably, two recent clinical trials emphasized the significance of the tailored vaccines in treating glioblastoma (56, 57). First, Keskin et al. treated glioblastoma patients with an individualized multi-epitope vaccine (NCT02287428), leading to increased neoantigen-specific CD4+ and CD8+ T cells responses in peripheral blood (56). Second, in another clinical trial, Hilf et al. reported that the combination of personalized vaccine and standard of care (NCT02149225) could induce sustained CD8+ T cells responses and predominantly CD4+ Th1 cell responses in glioblastoma patients (57).

Those encouraging results indicated that the personalized neoantigen vaccine approach is feasible for immunologically "cold" tumors with a low tumor mutation burden (56). Another early DC-based vaccine study in ovarian cancer has shown promising clinical outcomes without serious adverse events (59). Vaccination upregulated T cells responses against neoantigens, therefore elicited a broad antitumor immunity. In line with previous studies, the neoantigen-based EpiGVAX vaccine improved antitumor immunity in colorectal cancer (60). Recently, Ott et al. conducted a clinical trial that combined personalized neoantigen-based vaccine (NEO-PV-01) with nivolumab in patients with advanced melanoma, non-small cell lung cancer, and urothelial cancer (NCT02897765) (30). T cells responsive to neoantigens were detected in all vaccinated patients, while no serious adverse events were observed. The increasing T cells induced by this approach could control tumor growth and kill tumor cells, leading to potential clinical benefits (30).

To date, dozens of clinical trials investigating personalized neoantigen-based vaccines alone or in combination with checkpoint inhibitors are underway in various cancers (**Table 1**).

Adoptive Cell Therapies

Another neoantigen-targeted treatment approach is adoptive cell therapy (ACT). Natural or modified T cells are expanded *ex vivo* and infused into patients to enhance T cell responses and kill tumor cells. Adoptive T cell therapies include the adoptive transfer of TILs, or of T cells genetically engineered to express a T cell receptor (TCR), or a chimeric antigen receptor (CAR), as well as other immune cells like natural killer cells (61).

Personal TILs could recognize neoepitopes derived from somatic mutations were identified in gastrointestinal cancers (62). Several studies have shown that the adoptive transfer of T cells specific against oncogenic mutations could mediate tumor regression in metastatic cholangiocarcinoma (22), colorectal cancer (26), cervical cancer (63), and breast cancer (64). In 2014, Rosenberg et al. administrated neoantigen-reactive CD4+ TILs in a patient with metastatic cholangiocarcinoma (NCT01174121), resulting in complete tumor regression (22). This finding evidenced that CD4+ T cells against neoantigens can be used to mediate epithelial cancer regression. Then a patient with metastatic colorectal cancer was found to have CD8+ T cells in TILs which could specifically target mutant KRAS G12D (NCT01174121) (26). After infusion of the HLA-C*08:02–restricted TILs, all lung metastases' objective regression was observed. One lesion that progressed nine months later was proven to have the loss of chromosome 6 encoding the HLA-C*08:02 MHC class I molecule (26). Additionally, therapeutic TILs against mutant neoantigens induced immunodominant antitumor T cell responses instead

TABLE 1 | Clinical trials of neoantigen vaccines.

ClinicalTrial.gov identifier	Phases	Enrollment status	Cancer type	Vaccine format	Additional intervention	Patient accrual target
NCT03558945	Phase 1	Recruiting	Pancreatic tumor	peptide	None	60
NCT04487093	Phase 1	Recruiting	Non small cell lung cancer	peptide	EGFR-TKI/anti-angioge	20
NCT04397926	Phase 1	Recruiting	Non small cell lung cancer	peptide	None	20
NCT02950766	Phase 1	Recruiting	Renal cell carcinoma	peptide	Ipilimumab	19
NCT03359239	Phase 1	Recruiting	Urothelial cancer	peptide	Atezolizumab	15
NCT02287428	Phase 1	Recruiting	Glioblastoma	peptide	Pembrolizumab	56
NCT03956056	Phase 1	Recruiting	Pancreatic cancer	peptide	None	15
NCT04117087	Phase 1	Recruiting	Colorectal cancer, pancreatic cancer	peptide	Nivolumab, ipilimumab	30
NCT04248569	Phase 1	Recruiting	Hepatocellular carcinoma	peptide	Nivolumab, ipilimumab	12
NCT04072900	Phase 1	Recruiting	Melanoma	peptide	Toripalimab	30
NCT03953235	Phase 1/2	Recruiting	Solid tumors	peptide	Nivolumab, ipilimumab	144
NCT03639714	Phase 1/2	Recruiting	Solid tumors	peptide	Nivolumab, ipilimumab	214
NCT04161755	Phase 1	Recruiting	Pancreatic cancer	peptide	Atezolizumab, mFOLFIRINOX	20
NCT04024878	Phase 1	Recruiting	Ovarian cancer	peptide	Nivolumab	30
NCT03552718	Phase 1	Recruiting	Solid tumors	peptide	None	16
NCT04251117	Phase 1/2	Recruiting	Hepatocellular carcinoma	DNA	Pembrolizumab	24
NCT03199040	Phase 1	Recruiting	Triple negative breast cancer	DNA	Durvalumab	24
NCT04015700	Phase 1	Recruiting	Glioblastoma	DNA	None	6
NCT03674073	Phase 1	Recruiting	Hepatocellular carcinoma	DC	None	24
NCT04105582	Phase 1	Recruiting	Triple negative breast cancer	DC	None	5
NCT04078269	Phase 1	Recruiting	Non small cell lung cancer	DC	None	6
NCT04147078	Phase 1	Recruiting	Solid tumors	DC	None	80

EGFR, Epidermal growth factor receptor; TKI, Tyrosine-kinase inhibitor; DC, Dendritic Cell.

of against human papillomavirus (HPV) antigens, resulting in complete tumor regression in virally associated cervical cancer (63). Successful adoptive therapy was also found in patients with metastatic breast cancer (NCT01174121) (64). After received TILs against four neoantigens (SLC3A2, KIAA0368, CADPS2, and CTSB), the patient achieved durable tumor regression over 22 months (64). All these works supported that the adoptive transfer of neoantigen-based TILs played a vital role in immunotherapies.

Table 2 showed selected ongoing adoptive T cell therapy studies.

CHALLENGES

Despite recent advances, many challenges remain for the application of personalized neoantigen-based vaccine or adoptive cell transfer.

A critical issue that needs to be addressed is the expensive and time-consuming manufacturing. Although the cost of genome sequencing has decreased (65), it remains costly to identify neoantigens and process good manufacturing practices. The overall timeline from acquiring the patient's sample to vaccine administration was about 3 to 5 months (34). Reducing production turnaround time is urgent, especially for patients with metastatic disease. These cell-based experiments also are difficult to standardize and require significant numbers of cells. Thus high-throughput and unbiased computational strategies may need to select neoantigens (4). An additional obstacle could be neoantigens prediction and validation. Despite the current computational neoantigen prediction algorithms and experimental validation approaches (tetramers or multimers, ELISpot) used to prioritize neoantigens, some efforts are still needed to pursue further optimization. Strategies include better predict MHC-peptide binding and develop big datasets and new algorithms (4). In addition, tumor heterogeneity is common and can be caused by several factors, including 1) spontaneous mutations during tumor progression, 2) tumor microenvironments regulation or neoantigen loss (66), and 3) multiple lesions or even an individual tumor originated from different subclones (61). Tumor heterogeneity can reduce the accuracy of antigen clone prediction in heterogeneous tumor masses. Therefore, it is vital to analyze beneficial mutations carefully. In addition, fully personalized immunotherapy targeting multiple clonal neoantigens may also need to overcome the obstacle of tumor heterogeneity (4, 61). Another challenge is to define the reliable immune biomarkers to predict antitumor immunity and even survival benefit. Although immune-related response criteria (irRC) attempt to evaluate immunotherapeutic effects in clinical practice, they may still not fully reflect all the characteristics of clinical responses (67). Furthermore, the T cell responses induced by neoantigens-based therapies may not directly be translated into durable clinical responses. Thus, it is plausible to identify immune response biomarkers in a systematic pattern.

CONCLUSIONS

Emerging evidence reveals that tumor neoantigens play an essential role in antitumor immunity and successful cancer immunotherapies. Both personalized neoantigen-based

TABLE 2 | Clinical trial of neoantigen ACT.

ClinicalTrial.gov identifier	Phases	Enrollment status	Cancer type	Additional interventions	Patient accrual target
NCT03374839	Phase 1/2	Recruiting	Melanoma	Nivolumab, IL-2	11
NCT04072263	Phase 1/2	Recruiting	Ovarian cancer	Interferon alfa 2A, carboplatin, paclitaxel	12
NCT03709706	Phase 2	Recruiting	Non small cell lung cancer	Pembrolizumab	54
NCT02926053	Phase 1	Recruiting	Renal cell carcinoma	Fludarabine, cyclophosphamide, IL-2	6
NCT03967223	Phase 2	Recruiting	Solid tumors	Fludarabine, cyclophosphamide	65
NCT04555551	Phase 1	Recruiting	Multiple myeloma	None	36
NCT03970382	Phase 1	Recruiting	Solid tumors	Nivolumab, IL-2	148
NCT03754764	Phase 1/2	Recruiting	Lymphoblastic leukemia	None	80
NCT04526509	Phase 1	Recruiting	Solid tumors	Fludarabine, cyclophosphamide	38
NCT03720496	Phase 1	Recruiting	Non-Hodgkin lymphoma	None	6
NCT04217473	Phase 1	Recruiting	Melanoma	None	15
NCT02870244	Phase 1	Recruiting	Melanoma	None	18
NCT03497533	Phase 1/2	Recruiting	Non-Hodgkin lymphoma	None	6
NCT04430595	Phase 1/2	Recruiting	Breast cancer	None	100
NCT04571138	Phase 1/2	Recruiting	Leukemia, lymphoma	None	42
NCT04729543	Phase 1/2	Recruiting	Melanoma, head-and-neck carcinoma	None	20
NCT04596033	Phase 1	Recruiting	Solid tumors	Fludarabine, cyclophosphamide, IL-2	24
NCT00881920	Phase 1	Recruiting	Lymphoma, myeloma, leukemia	None	54
NCT03638167	Phase 1	Recruiting	Pediatric central nervous system tumor	None	36
NCT03500991	Phase 1	Recruiting	Pediatric central nervous system tumor	None	48
NCT03326921	Phase 1	Recruiting	Acute Leukemia	Fludarabine	24
NCT03318900	Phase 1	Recruiting	Ovarian carcinoma	Cyclophosphamide, utomilumab, IL-2	18
NCT03412526	Phase 2	Recruiting	Ovarian Cancer	Fludarabine, IL-2	15
NCT03338972	Phase 1	Recruiting	Multiple myeloma	Fludarabine, cyclophosphamide	25
NCT03186118	Phase 1	Recruiting	Acute Leukemia	None	30
NCT03166878	Phase 1/2	Recruiting	Leukemia, Lymphoma	None	80
NCT03330691	Phase 1	Recruiting	Leukemia	None	46
NCT02028455	Phase 1/2	Recruiting	Leukemia	None	80
NCT03823365	Phase 1	Recruiting	Non-Hodgkin lymphomas, chronic lymphocytic leukemia	None	13
NCT01955460	Phase 1	Recruiting	Melanoma	Fludarabine, cyclophosphamide, IL-2	15
NCT03434769	Phase 1	Recruiting	Non-Hodgkin lymphoma	Fludarabine, cyclophosphamide	18

vaccines and ACT approaches have shown encouraging antitumor results. Decreased production turnaround time, reduced manufacturing cost, better detection of immunogenic neoantigens, improved computational algorithms, and effective treatment biomarkers are expected to add the feasibility, affordability, and momentum of neoantigen targeting therapies. Neoantigen-based therapies have the potential to turn "cold" tumors into "hot" ones. Therefore, it's warranted to explore the combinatorial approaches with other immunotherapies, including checkpoint blockade therapies or conventional treatments, including chemoradiotherapies, kinase inhibitors, anti-angiogenesis therapies, et al. (68). Thus, it's plausible to think that neoantigen-based tailored therapies can be widely performed in various cancers soon.

AUTHOR CONTRIBUTIONS

YZ: Conceptualization, writing the original draft. JL: Funding acquisition, writing review, and editing. All authors contributed to the article and approved the submitted version.

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Prognostic Value of Neoantigen Load in Immune Checkpoint Inhibitor Therapy for Cancer

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Immune checkpoint inhibitors (ICIs) have made great progress in the field of tumors and have become a promising direction of tumor treatment. With advancements in genomics and bioinformatics technology, it is possible to individually analyze the neoantigens produced by somatic mutations of each patient. Neoantigen load (NAL), a promising biomarker for predicting the efficacy of ICIs, has been extensively studied. This article reviews the research progress on NAL as a biomarker for predicting the anti-tumor effects of ICI. First, we provide a definition of NAL, and summarize the detection methods, and their relationship with tumor mutation burden. In addition, we describe the common genomic sources of NAL. Finally, we review the predictive value of NAL as a tumor prediction marker based on various clinical studies. This review focuses on the predictive ability of NAL's ICI efficacy against tumors. In melanoma, lung cancer, and gynecological tumors, NAL can be considered a predictor of treatment efficacy. In contrast, the use of NAL for urinary system and liver tumors requires further research. When NAL alone is insufficient to predict efficacy, its combination with other indicators can improve prediction efficiency. Evaluating the response of predictive biomarkers before the treatment initiation is essential for guiding the clinical treatment of cancer. The predictive power of NAL has great potential; however, it needs to be based on more accurate sequencing platforms and technologies.

Keywords: cancer, neoantigen load, immune checkpoint inhibitor, biomarker, prognostic value

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INTRODUCTION

Tumors acquire mutations as they develop and progress. These mutations can encode amino acid sequences to translate different proteins, called tumor-specific antigens or neoantigens, which are immunogenic and can be recognized and eliminated by immune cells (1, 2). Compared with traditional chemotherapy and targeted therapy, immune checkpoint inhibitors (ICIs) therapy have an enduring effect and efficacy in relieving the inhibitory effect of tumor cells on immune cells, thus enhancing the immune response to cancer cells. Neoantigens elicit T-cell immunoreactivity and sensitivity to ICIs (3).

Studies have shown that new epitopes avoid the effect of central T-cell tolerance, and endogenous T cells recognize new epitopes and eliminate them, making neoantigens a promising target for cancer immunotherapy (4–6). Currently, new sequencing technologies, specialized calculation methods, and the combination of human leukocyte antigen (HLA) are used to predict neoantigen load (NAL). A certain correlation between NAL and disease prognosis is an intrinsic property of neoplasms (7). A preclinical study using a UVB-induced mouse melanoma model reported that high NAL levels can predict the response probability of ICIs (8). A series of follow-up clinical studies have shown that higher NAL is associated with enhanced efficacy of ICIs in melanoma, non-small cell lung carcinoma (NSCLC), and colorectal cancer (6, 9–11).

These studies on NAL provide new directions for individualized immunotherapy. Herein, we review patients who were responsive to ICIs and had tumors with NAL expression. This review provides new insights on prognostic and predictive biomarkers of ICI sensitive cancers.

DEFINITION OF NAL

Tumor-specific expression, such as that of somatic mutations, alternative splicing, fusion genes, non-coding RNA, and circular RNA, may produce tumor-specific antigen polypeptides (12–15). The formation of new antigens requires several steps. First, the polypeptide enters the endoplasmic reticulum (ER) through a transporter associated with the antigen processing (TAP) complex. In the ER, these peptides bind to major histocompatibility complex (MHC) class I molecules with different affinities, and the peptide-MHC class I complex is transported to the plasma membrane through the Golgi complex and recognized by CD8+ cytotoxic T cells. Although T cells can recognize antigens shared by normal and tumor cells, T-cell receptors (TCRs) usually have a higher affinity for neoantigens (16, 17). Some mutant proteins can be recognized by TCRs as neoantigens, resulting in the initiation of an immune response. The reactivity of TCRs expressed by tumor-infiltrating lymphocytes (TILs) determines their ability to interact with tumor antigens on antigen-presenting cells (APCs). Therefore, the TCR library is related to the response and survival of cancer patients to immune checkpoint blockade therapy (18, 19).

RELATIONSHIP BETWEEN NAL AND TUMOR MUTATION BURDEN

The tumor mutation burden (TMB) generally refers to the number of non-synonymous mutations per megabase (Mb) of somatic cells in a specific genomic region TMB can be used to estimate the ability and tumors to produce new antigens, and has been proven to predict the efficacy of immunotherapy for a variety of tumors (1, 20). In the past, whole-exome sequencing (WES) was the first choice for TMB detection, accounting for 1% of the entire genome, including most known pathogenic mutations (21). However, its application in clinical practice is limited because of its high cost, large sample demand, and complex data analysis (20, 22–24). With the identification of a large number of tumor-related genes, the use of targeted sequencing panels for tumor genome analysis has become another option in clinical testing.

Early screening of new antigens was mainly performed using a cDNA library, however, this is a very time-consuming and laborious process. With the development of WES (25), whole-genome sequencing (26), and second-generation sequencing of the transcriptome (27), the cost of sequencing has been dramatically reduced, making it possible to quickly and effectively perform individual sequencing and neoantigen screening for each patient, thereby laying the foundation for the clinical application of NAL. In addition, large projects, such as The Cancer Genome Atlas (TCGA) (25, 28) and the International Cancer Genome Consortium (29), have identified cancer genomes across multiple tumor types. Directly excavating tumor neoantigenic epitopes in the databases and literature can identify high-frequency mutation sites in solid tumors. In addition, single-cell sequencing methods have been increasingly adopted as a high-resolution alternative method to study gene expression, genomic aberrations, microenvironment, and epigenetic modifications in the constituent cells of various malignant and benign tumors (30, 31). Overall, neoantigens play a pivotal role in cancer immunotherapy, especially in ICI therapy. The key to achieving high effective individualized immunotherapy is the development of new bioinformatics and calculation methods to improve the sensitivity and specificity of antigen identification methods.

TMB has been used as a target for predicting the efficacy of ICI therapy. Theoretically, tumor types with a high TMB often have a high predictive NAL (32). The relationship between overall TMB/NAL and ICI response in NSCLC and melanoma has been clarified in various studies (6, 9, 11). The primary explanation is that high TMB increases the formation and presentation of immune neoantigens, thereby inducing effective anti-tumor immune responses (33). Recent studies have confirmed that the higher the TMB, the higher the tumor NAL, and the more likely to a patient benefits from ICI therapy (34). It is speculated that tumors with higher mutation burden have more tumor-specific neoantigens, which stimulate the increase in the number of TILs caused by the overexpression offset of immune checkpoint modulators, such as the programmed death receptor 1 (PD-1) or programmed cell death ligand 1 (PD-L1) (35–38). ICIs can promote T cells to recognize tumors by antagonizing T-cell activation inhibitory molecules, thereby restoring the anti-tumor immune response (39).

However, TMB is not equivalent to NAL. Rizvi et al. showed that the absolute burden of candidate neoantigens, but not the frequency per non-synonymous mutation, correlated with response, suggesting the importance of neoantigens in dictating response (6). Another study analyzed the different patterns of TMB and NAL numbers in NSCLC and found that half of the oncogenic mutations did not produce neoantigens, suggesting that TMB number is not a good surrogate marker of the

immunogenic neoantigen (40). TMB may thus be an indirect measure of tumor immunogenicity because somatic mutations must lead to amino acid changes in expressed proteins; thus, peptides must be presented by HLA and subsequently cause cell proliferation and kill tumors. Studies aiming to evaluate and improve the prediction of NAL when high TMB cannot effectively predict benefit from ICI therapy should be conducted in the future.

NAL AND GENOMIC ALTERATIONS

Currently, a large number of clinical trials have explored whether gene mutations can be used to estimate NAL to predict the response of various cancers to ICIs. The relationship between common oncogenes and NAL has been explored. Driver mutant genes may interfere with genome stability and affect immune status by generating new antigens (41). One study reported that the number of predicted neoantigens was significantly higher in BRCA1/2 mutant tumors and that tumors with higher NAL were associated with improved overall survival (OS) and higher expression of immune genes associated with tumor cytotoxicity (42). Another study found that patients with mutant TP53 (TP53-MT) showed stronger tumor antigenicity and tumor antigen presentation than patients with wild-type TP53 (TP53-WT) and were more likely to benefit from ICI therapy (43). Tran et al. found KRAS G12D mutations in lung metastasis resection tissues of patients with rectal cancer and detected polyclonal CD8+ T cells that specifically recognize KRAS G12D mutations in TILs (44).

Some rare gene mutations can also cause an increase in NAL; however, findings on the prognosis remain inconclusive. For example, Lei Zhang et al. reported that compared with patients with wild-type tumors, patients with MUC16 mutant tumors have a significant increase in NAL, which is related to improved the OS of patients with MUC16 mutation containing NSCLC and melanoma (45). Similarly, Wu et al. conducted a comprehensive analysis of patients with TET1, a DNA demethylase that regulates DNA methylation (46). They indicated that TET1 mutation was closely associated with higher NAL, presenting a higher objective response rate, better durable clinical benefit, longer progression-free survival (PFS), and improved OS in patients receiving ICIs (47). A series of studies have shown the relationship between genetic mutations and NAL, such as TP53-MT (43), Eph receptor A5 mutations (48), ZFHX3-MT (49), and AT-rich interaction domain 1A (50), which are closely related to longer OS or PFS in patients treated with ICIs.

The DNA damage response system is essential for the preservation of genomic integrity (51), and thus, mutations in this system may lead to the appearance of new alleles that are absent in normal DNA, resulting in an increase in NAL. Wang et al. demonstrated that variations in the DNA damage response pathway of homologous recombination repair (HRR), mismatch repair, and base excision repair are associated with increased NAL and increased levels of immune gene expression characteristics (52). Similarly, a study reported that a deficiency in DNA double-strand break repair, particularly HRR, is related to increased NAL on the tumor cell surface, which subsequently activates the adaptive immune response (53).

In addition, other markers can represent an increase in NAL and can thus be used as predictors of ICI efficacy. For example, the centrosome protein 78 (CEP78) is required to regulate the cell cycle (54). Huang et al. analyzed the RNA sequencing data of a muscle-invasive bladder cancer cohort and found that high CEP78 expression was correlated with high NAL; but was not associated with OS (55). A similar study has shown that the high expression of the aryl hydrocarbon receptor nuclear translocator-like protein 1 is associated with increased NAL and can be a clinically relevant biomarker for immunotherapy (56). A low m6A score was also linked to increased NAL and enhanced response to immunotherapy (57).

As more mutated genes are revealed to be clearly associated with an increase in NAL, cancer patients can be better screened for ICI therapy.

PROGNOSTIC VALUE OF NAL IN TUMORS

The commonly used targets of immunotherapy are mainly cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or PD-1/PD-L1, which can effectively treat a variety of malignant tumors (58). Presently, it is generally believed that neoantigens and neoantigen-specific T cells are closely related to tumor regression after ICI therapy. Tumors with more mutations may produce more new epitopes, which can be recognized by tumor-infiltrating T cells. Checkpoint blocking antibodies activate these T cells in the body and induce tumor regression (59) (**Figure 1**).

These mechanisms promote the success of ICI therapy and NAL through several aspects. Tumors containing DNA repair gene mutations, such as *HR* gene, *MMR* gene, or POLE mutations, have a significantly higher mutation load and a significantly greater number of T-cell types and other anti-tumor activities than DNA repair wild-types tumors. The required immune cell infiltration increases the efficacy of ICI therapy (60). Zhu et al. analyzed the mechanism underlying the improved prognosis of NAL-high (NAL-H) and NAL-medium (NAL-M) groups. They found that the high- and medium-expression groups of NAL have significantly overexpressed genes, which are related to IFN-g/TNF-α, and are important predictors of immune activation (61). In addition, these two groups of patients have a higher degree of adaptive immune infiltration, whereas the low-expression group is enriched with innate immune infiltration. A series of studies have shown that the level of NAL can help screen this part of the patients (6, 42, 60–74) (**Table 1**).

NAL in Melanoma

The emergence of ICIs has completely changed the clinical management of metastatic melanoma, which has a higher mutation burden than other solid tumors (75). A clinical trial that included 110 patients with metastatic melanoma who were treated with CTLA-4 inhibitors revealed the correlation between NAL and clinical outcome, indicating that NAL could be used as a potential biomarker patients selection (62). Another study that focused on the detection of somatic mutations and the transcriptome of metastatic melanoma to identify the factors

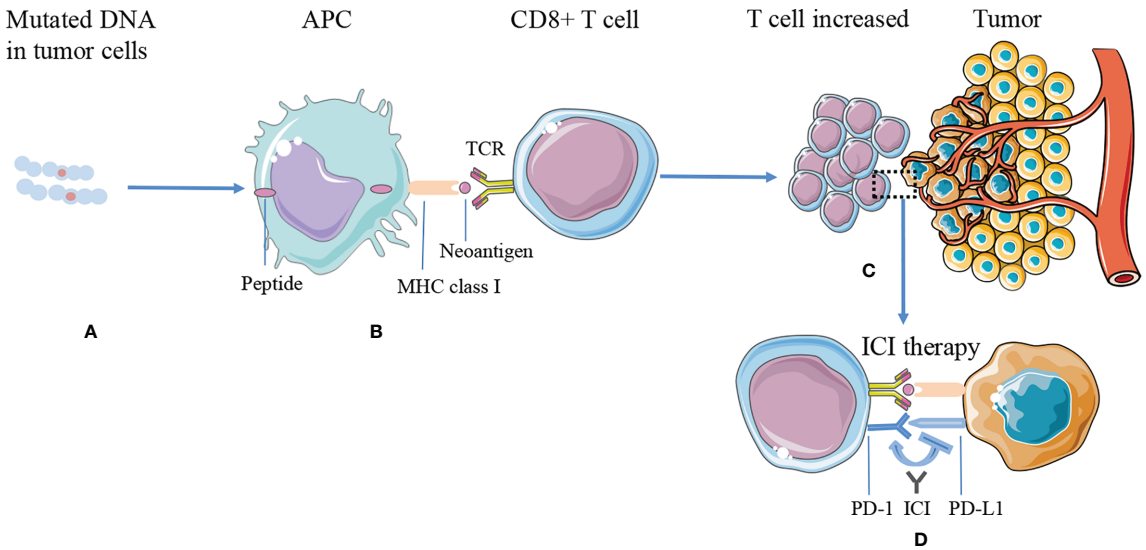

FIGURE 1 | The mechanism of tumor antigen processing, presentation on MHC class I, and improving efficacy of ICI therapy. **(A)** DNA mutations occurred and synthesized proteins in the tumor cells. **(B)** The proteins are processed into smaller peptides, displayed by major histocompatibility complex (MHC) class I molecules *via* APC cells, and recognized by CD8+ T effector cells as neoantigens. **(C)** Tumors expressing higher numbers of neoantigens are more likely to induce a significantly greater number of T cells, while tumor cells inhibit T-cell function through immune checkpoints, such as PD-L1. **(D)** ICI therapy blocks immune checkpoint suppression, reactivates T-cell function, and kills tumor cells. APC, antigen-presenting cell; ICI, immune checkpoint inhibitor; MHC, major histocompatibility complex; PD-L1, programmed cell death ligand 1; PD-1, programmed death receptor 1; TCR, T-cell receptors.

that may affect anti-PD-1 therapy found no statistical difference in NAL between reactive and non-reactive tumors; thus, the NAL of melanoma tumors before treatment was insufficient to predict the response to anti-PD-1 therapy (63).

NAL in Lung Cancer

Neoantigens are associated with the response to anti-PD-1 therapy in patients with NSCLC (6). A previous study analyzed mutations in DNA repair genes using TCGA samples and found that NAL correlated with the expression of PD-1, PD-L1, and IFN-γ and tended to increase the OS of patients with lung adenocarcinoma. NAL is linked to DNA repair mutations, increased number of TILs, and favorable survival outcomes (60). Furthermore, advanced NSCLC treated with PD-1 and CTLA-4 blockade-based therapies was profiled for intra-tumor heterogeneity (ITH) and neoantigen burden. High NAL was associated with significantly longer OS in patients with lung adenocarcinoma. Notably, patients with homogeneous tumors (neoantigen ITH ≤1%) have a prolonged OS compared with those with heterogeneous tumors (64). Rizvi et al. analyzed NSCLC samples collected from patients treated with pembrolizumab and reported that higher NAL in tumors was associated with improved objective response, durable clinical benefit, and PFS (6).

NAL in Gynecologic Cancer

Immunotherapy is also widely used in the field of gynecological tumors, and identifying the ideal predictive markers for therapeutic efficacy has always been the goal of researchers. A recent study developed a new informatics workflow, which was applied to detect class I and class II HLA-bound neoantigens, and reported the association between NAL and OS in breast cancer (65). A clinical study including 812 gynecologic and breast cancer patients used a NAL cutoff of 60% and 80% and divided the patients into three groups. It was found that the NAL-high and NAL-middle groups had a higher number of T cells, B cells, and cytotoxic lymphocytes, whereas the NAL-low group was rich in eosinophils, NK cells, mast cells, and interdigital cells, which represent adaptive immunity and innate immunity, respectively. Furthermore, the NAL-high group was associated with better OS, higher immune infiltration, and lower intratumoral heterogeneity (61).

A previous study showed that hypermutated POLE-mutated endometrial cancer has a higher predictive NAL and is related to its prognosis (76). Shukla et al. investigated whether low-mutation endometrial cancer has similar prognostic factors and analyzed the data of 90 copy number-low/endometrioid and 60 copy number-high/serous-like endometrial tumors using the TCGA dataset. They found that the predicted NAL was related to specific genomic changes, such as CTNNB1 mutation, MYC amplification, and PIK3CA mutation. In copy number-low/endometrioid tumors, high NAL was associated with prolonged PFS, and low NAL in serous-like endometrial tumors was associated with poor PFS (66).

Deficiencies in the homologous recombination (HR) pathway are common in high-grade serous ovarian cancer (77, 78). The best candidates for ICIs in HR-proficient ovarian cancer patients who cannot benefit from poly(ADP-ribose) polymerase inhibition have been investigated. In particilar, the exome and RNA sequencing data of 80 patients with high-grade serous ovarian cancer were

TABLE 1 | Studies describing the impact of neoantigen load evaluation in the clinical research.

Type of cancer	No of investigated patients	Test for NAL	Group	Drug/treatment	Result (whether NAL is associated with clinical benefit)	Reference
Melanoma	110	WES	High/low	Ipilimumab	YES	(62)
Melanoma	38	WES	High/low	Pembrolizumab and nivolumab	NO	(63)
Non–small cell lung cancer	34	WES	High/low	Pembrolizumab	YES	(6)
Non–small cell lung cancer	NR	RNA-sequencing	High/low	NR	YES	(60)
Non–small cell lung cancer	139	NR	High/low	PD-1 and CTLA-4 blockade	YES	(64)
Breast cancer	835	WES and RNA sequencing	high/ medium/ low	NR	YES	(65)
Gynecologic and breast cancers	812	RNA-sequencing	high/ medium/ low	Immunotherapy	YES	(61)
Endometrial cancers	150	WES	High/low	Immunotherapy	YES	(66)
Ovarian carcinoma	80	WES and RNA sequencing	High/low	Carboplatin plus paclitaxel	YES	(67)
Ovarian cancer	253	WES	High/low	PD-1/PD-L1 inhibitors	YES	(42)
Bladder tumors	37	RNA sequencing	High/low	Durvalumab	YES	(68)
Muscle-invasive Bladder Cancer	38	WES	High/low	NR	NO	(69)
Clear cell renal cell carcinoma	97	WES and RNA sequencing	High/low	Surgery alone or surgery plus cytokines tyrosine kinase inhibitors and mTOR inhibitors	NO	(70)
Clear cell renal cell carcinoma	592	WES and RNA sequencing	High/low	PD-1 blockade	NO	(71)
Multiple myeloma	184	WES and RNA sequencing	High/low	Chemotherapy, or immunotherapy	YES	(72)
Osteosarcoma	321	WES	High/low	Pembrolizumab	YES	(73)
Hepatocellular carcinoma	22	WES and RNA sequencing	High/low	Surgery alone or surgery plus chemoradiotherapy	NO	(74)

CTLA-4, cytotoxic T-lymphocyte-associated protein 4; NAL, neoantigen load; NR, not reported; PD-1, programmed death receptor 1; PD-L1, programmed cell death ligand 1; WES, whole-exome sequencing.

analyzed. They found that the OS and PFS of the high-NAL and low-NAL groups were not statistically different. However, the inclusion of HLA class I expression status in the survival analysis showed that the subgroup of patients with high NAL and high HLA class I expression had the best PFS in HR-proficient high-grade serous ovarian cancer patients (67). Similarly, in ovarian carcinoma patients, early findings suggested that NAL is significantly associated with OS but not with PFS (42).

NAL in Urothelial Cancer

Urothelial cancer has a high burden of somatic mutations, second only to lung cancer and melanoma (79). A study described a systematic method to effectively identify and verify immunogenic neoantigens. This method was verified in some patients with bladder tumors who received durvalumab treatment. In this cohort, the most predicted neoantigen in all patients was immunogenic *in vitro*. Finally, the patients were stratified by TMB or NAL using the three-point method to evaluate OS. The results showed that patients with higher NAL showed better OS. Although the number of included cases was small, the study demonstrated the predictive value of ICI therapy on bladder cancer; thus, future studies should examine lager cohorts (68). However, in a study of 38 muscle-invasive bladder cancer tissues from patients who underwent definitive surgery. Choudhoury et al. found that the relationship between filtered NAL and recurrence-free survival (RFS) was not statistically significant (69). Two other studies in clear-cell renal cell carcinoma have similar reported that NAL is not associated with response to ICI therapy (70, 71).

NAL in Other Cancer

Mutations and NAL are associated with prolonged survival in patients with newly diagnosed multiple myeloma (80). In a recent study, researchers used next-generation sequencing data to describe the distribution of neoantigens in multiple myeloma and found that in patients with multiple myeloma recurrence when compared with newly diagnosed multiple myeloma patients. In this study, the neoantigen T-cell response of three patients with multiple myeloma recurrence, the NAL increased was verified and correlated with improved clinical response (72). Osteosarcoma often presents with lung metastases, and there is a lack of effective treatment strategies for it (81). Researchers have sequenced the multi-region whole exome and whole genome of 86 tumor regions of lung metastatic osteosarcoma. Metastatic tumors showed better immunogenicity, higher NAL, higher PD-L1 expression, and more TILs than primary tumors. One patient relapsed after the first primary tumor operation and subsequent lung metastasis resection. After

multiple chemotherapy regimens, the patient received six cycles of pembrolizumab treatment. Lung metastases showed a partial response, and some lung metastases had disappeared, thus demonstrating that NAL may also be a potential biomarker for lung metastatic osteosarcoma (73). Yang et al. investigated neoantigens in hepatocellular carcinoma and concluded that OS was not associated with NAL (74).

LIMITATIONS OF NAL IN CLINICAL SETTINGS

In general, ICI therapy is not effective for all patients, and the relationship between NAL and clinical outcomes is not consistent among cancer types. However, the exact mechanisms responsible for such differences remain unclear. Here, we will disscuss some of these reasons. First, the rapid increase in the heterogeneity of tumor cells may lead to the failure of immune monitoring, thereby resulting in non-response to immunotherapy (64, 82–84). Tumors with low neoantigen ITH are associated with longer PFS (64). One possible explanation for this is that tumors with high ITH may have more neoantigens, which are subsequently presented by DCs to T cells in the form of MHC class I peptide complexes. Eventually, the ITH level in these tumors changes from high to low (85). Second, most previous studies did not comprehensively analyze the mutation types, which would result in missing data and inaccurate results. For example, they mostly included analysis of somatic non-synonymous single nucleotide mutations and small frameshift insertions and deletions but did not consider large genome rearrangements or gene fusions. The contribution of fusion genes exceeds one-third of the total NAL, and there is no correlation between gene fusion NAL and OS. Thus, this will have a significant impact on the results if there is no sufficient analysis of mutation types (65). Third, most of the analyzed data are derived from the TCGA database, wherein tumor samples are screened and excluded by pathologists. This results in a loss of information, which will inevitably affect the follow-up results (86). In addition, some researchers have proposed that the ability of neoantigens to activate T-cell recognition and the quality of T-cell responses are more important in determining the immune response during tumor evolution than the number of neoantigens. Therefore, the quality rather than the quantity of neoantigens may that affect the efficacy of ICI therapy (84). Some studies with insufficient sample sizes may not have reliable conclusions.

In addition, changes in the tumor genome landscape during ICI therapy may lead to the possible evolution of NAL and affect the efficacy of ICI therapy. Some patients who initially responded to PD-1 blockade therapy developed resistance (87). Alternate upregulation of immune checkpoints (88), loss of HLA haplotypes (89), and somatic mutations in *HLA* or *JAK1/JAK2* genes (90, 91) have been considered mechanisms by which some patients evade immune recognition. Using comprehensive genomic analysis, it was determined that the emergence of acquired drug resistance during immune checkpoint blockade therapy is related to the mutation and loss of putative tumor-specific neoantigens, including the elimination of tumor subclones or truncated changes in chromosomes (92). Several recent studies have shown that the possible mechanism for the change in the neoantigen mutation landscape during ICI treatment is the induction of tumor resistance by losing antigen or components of the antigen presentation pathway, such as b-2 microglobulin (13, 44, 91, 93, 94). Simultaneous targeting of multiple antigens or MHC class II-restricted antigens can overcome this resistance. In addition, the combination of checkpoint blocking therapy and T-cell therapy can prevent T-cell failure and improve clinical efficacy (95).

To more accurately predict the therapeutic effect, NAL combined with other indicators is a feasible method of testing. A recent study reported no difference between the high-NAL and the low-NAL groups; however, according to the subgroup analysis results, the high-NAL and the high- HLA-I expression groups were associated with better PFS than the other groups (67). Similarly, in a clear-cell renal cell carcinoma cohort analyzed by Matsushita et al., the high level of NAL combined with the number of HLA-restricted neoepitopes correlated with better clinical outcomes (70). Another study demonstrated that NAL was not correlated with RFS, however, patients with more neoantigens and low T-cell receptor β diversity had a prolonged RFS compared with those with fewer neoantigens and high TCR diversity (69). Therefore, when NAL alone cannot be used to predict efficacy, the combined test will facilitate the application of NAL and improve prediction efficiency.

CONCLUSIONS

ICIs provide cancer patients with more options, in addition to targeted therapy drugs. However, the effectiveness of this treatment is not satisfactory and many patients do not benefit from it. The exploration of effective curative predictors is currently ongoing, and NAL has a promising as a new generation of ICI biomarkers. With rapid advancements in sequencing technologies, NAL can become more reliable markers. NAL alone or in combination with other indicators can provide accurate clinical guidance for patients receiving immunotherapy.

AUTHOR CONTRIBUTIONS

X-bL and W-yC: conceptualization. X-lZ, G-yZ, and HK: original draft writing. JY, QT, C-jZ, J-lZ, and RZ: manuscript review and editing. All authors contributed to the article and approved the submitted version.

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