

Targeting Neoantigens in Hepatocellular Carcinoma for Immunotherapy: A Futile Strategy?

Ligong Lu,¹ Jun Jiang,² Meixiao Zhan,¹ Hui Zhang,³ Qian-Ting Wang,² Sheng-Nan Sun,² Xiao-Kai Guo,² Hua Yin,¹ Yadong Wei,⁴ Jun O. Liu,⁴ Shi-You Li,² Yong Li,¹ and You-Wen He⁵

Hepatocellular carcinoma (HCC) is one of the most lethal cancer types, and treatment options for patients with advanced-stage HCC are limited. Neoantigens are newly expressed antigens on tumor cells that may derive from viral proteins or unmutated or mutated cellular proteins.⁽¹⁾ Technical advances in high-throughput genomic and proteomic sequencing and the development of algorithms to predict major histocompatibility complex (MHC) class I and II neoepitopes have made personalized cancer immunotherapy targeting neoantigens a reality.⁽²⁾ With the recent surge of interest in neoantigens as immunotherapeutic targets, several clinical trials have been initiated to test neoantigen-based therapy in HCC. More than 100 clinical trials are registered at ClinicalTrials.gov to test neoantigen vaccines for different solid tumors. However, recent findings suggest that neoantigen-based immunotherapy for HCC and other solid tumors faces major challenges.

Recent Clinical Application of Neoantigens as Therapeutic Targets

Heralded as a new era in cancer immunotherapy, neoantigen-based therapy is advancing rapidly.⁽²⁾ One clinical approach to target neoantigens in cancer cells is to adoptively transfer neoantigen-specific T cells from *in vitro*-expanded tumor-infiltrating lymphocytes (TILs). Impressive clinical efficacy was achieved after neoantigen-specific T-cell transfer into a patient with metastatic cholangiocarcinoma⁽³⁾ and extended to other solid tumors.⁽⁴⁾ However, technical issues need to be resolved before the wide application of the adoptive transfer of neoantigen-recognizing T cells in cancer treatment. The tumor materials procured for TIL expansion in patients with advanced-stage cancer produce a variable quality and quantity of neoantigen-specific TILs because of the high intratumor heterogeneity (ITH) of the T-cell

Abbreviations: Cas9, CRISPR-associated 9; CD, cluster of differentiation; CRISPR, clustered regularly interspaced short palindromic repeats; HCC, hepatocellular carcinoma; HLA, human leukocyte antigen; ITH, intratumor heterogeneity; MHC, major histocompatibility complex; MMR, mismatch repair; PD-1, programmed cell death 1; TAA, tumor-associated antigen; TAP, transporter associated with antigen processing; TIL, tumor-infiltrating lymphocyte; TMB, tumor mutation burden.

Received January 21, 2020; accepted April 2, 2020.

Supported by the National Key Research and Development Program of China (2017YFA0205200), the National Natural Science Foundation of China (81571785, 81771957, 81901857, and 81801811), the Natural Science Foundation of Guangdong Province, China (2018A030313074, to L.L.), and the Sunny Li Family fund (to Y.-W.H.).

© 2020 by the American Association for the Study of Liver Diseases.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.31279

Potential conflict of interest: Dr. He owns stock in Tricision and TriVac. Dr. S. Li is employed by and owns stock in Tricision. Dr. Liu, owns stock in, and holds intellectual property rights with TriVac.

receptor repertoire. In addition, the associated time and cost are extensive, and the success rates are low.⁽⁴⁾

A second clinical approach to target neoantigens for cancer immunotherapy is to identify neoepitopes from tumor samples and vaccinate patients with neoantigen-based cancer vaccines. Clinical studies employing personalized neoantigen peptide, mRNA, or dendritic cell vaccines in patients with melanoma showed encouraging neoantigen-specific T-cell induction and clinical efficacy.⁽⁵⁻⁷⁾ This approach has also been tested in glioblastoma, a low tumor mutation burden (TMB) cancer type.^(8,9) In one study, 8 patients received peptide vaccines of *in silico*-identified neoantigens and standard radiotherapy after surgery.⁽⁸⁾ Although neoantigen-specific T-cell responses were induced in the immunized patients, the median overall survival (OS) and 2-year survival were not superior (with the caveat of a small number of patients) to those achieved by standard adjuvant treatment.⁽¹⁰⁾ There may be many reasons that this vaccine could have been ineffective, such as the unmethylated O⁶-methylguanine-DNA methyltransferase promoter status in all patients or the median time of 19.9 weeks to first vaccination administered after surgery; however, a fundamental issue limiting the efficacy of neoantigen vaccines in glioblastoma may be the rare expression of neoantigens on the surface of glioblastoma cells by MHC molecules.⁽⁹⁾ Thus, although neoantigen vaccines have induced specific T cells in patients, these T cells cannot recognize tumor cells lacking neoepitope presentation.

In a second study, 15 patients with glioblastoma received two types of personalized peptide vaccines in addition to standard chemoradiotherapy after surgery.⁽⁹⁾ One vaccine contained unmutated tumor-associated antigens (TAAs) and cellular proteins identified from the human leukocyte antigen (HLA) immunopeptidomes by mass spectrometry. The second vaccine employed neoantigens identified *in silico*. Although the initial protocol planned to use mass spectrometry-based HLA immunopeptidome analysis to identify mutated neoepitopes, none of the 643 genomic mutations were detected in the HLA peptidomes of the individual patients⁽⁹⁾ (Table 1). In contrast, mutated neoepitopes were identified in patients with hypermutated glioblastoma,⁽⁹⁾ demonstrating the sensitivity of this strategy. The clinical trial presented a median OS of 29.0 months and a 2-year survival rate of 50% (8 of 16), which outperformed standard adjuvant radiochemotherapy.⁽¹⁰⁾ The different clinical outcomes between these two trials may suggest a strategy of combining TAAs and neoantigens in personalized vaccines. However, the favorable efficacy in the second trial may be due solely to personalized TAA vaccines. The fact that none of the 643 genomic mutations were detected as HLA-presented neoepitopes suggests that these neoantigens are rarely expressed on the surface of individual tumors, and their roles in antigen-specific T cell-mediated tumor rejection remain unclear.

ARTICLE INFORMATION:

From the ¹Zhuhai Interventional Medical Center, Zhuhai Precision Medical Center, Zhuhai People's Hospital, Zhuhai Hospital Affiliated with Jinan University, Zhuhai, P.R. China; ²Tricision Biotherapeutic Inc., Zhuhai, P.R. China; ³First Affiliated Hospital, China Medical University, Shenyang, P.R. China; ⁴Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD; ⁵Department of Immunology, Duke University Medical University Medical Center, Durham, NC.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

You-Wen He, M.D., Ph.D.
Department of Immunology
Duke University Medical University Medical Center
Box 3010, DUMC
Durham, NC 27710
E-mail: youwen.he@duke.edu
Tel.: 9194506096
or

Ligong Lu, M.D.
Zhuhai Interventional Medical Center
Zhuhai Precision Medical Center
Zhuhai People's Hospital
Zhuhai Hospital Affiliated with Jinan University
No. 79, Kangning Road
Xiangzhou District, Zhuhai, 519000, Guangdong Province, China
E-mail: luligong1969@126.com
Tel.: 86 (20) 8522-0610

TABLE 1. Identification of Neoepitopes in HCC and Other Tumors by Mass Spectrometry and Cancer Genomics

	HCC	Melanoma	Glioblastoma	Colorectal Cancer	Cholangiocarcinoma
Number of patients	16	5	15	5	1
DNA mutations	1,039	50-1,200*	643	612	54
Unique peptides	1,403*	95,662†	NA	9,936	1,318
Neoepitope	0	11	0	3	0
TMB rank‡	12th	1st	22nd	7th	12th
Number of clonal					
Neoantigens rank§	8th	1st	14th	3rd	NA

References for HCC,⁽¹³⁾ melanoma,⁽¹²⁾ glioblastoma,⁽⁹⁾ colorectal cancer,⁽¹⁹⁾ cholangiocarcinoma,⁽²⁰⁾ TMB rank,⁽¹⁴⁾ and clonal neoantigen rank.⁽³²⁾

*Number per patient.

†Total number of unique peptides from 25 patient samples.

‡The median number of tumor mutations in 30 different cancers was ranked. Liver cancer is listed as a reference for HCC and cholangiocarcinoma as intrahepatic cholangiocarcinoma has a similar mutation rate to that of HCC.⁽⁴⁵⁾

§The median number of clonal neoantigens in 16 different cancers was ranked.

Abbreviation: NA, not available.

Three Levels of Neoantigen Identification Strategies in Clinical Application: Feasibility and Pitfalls

Neoantigens serving as effective immunotherapeutic targets need to both have strong immunogenicity to induce potent antigen-specific cluster of differentiation 4-positive (CD4⁺) and CD8⁺ T cells and be presented (ideally abundantly) by MHC class I and II molecules on the tumor cell surface. These two features of neoantigens, immunogenicity and targetability, ensure the recognition and efficient lysis of tumor cells by vaccine-induced or *ex vivo*-expanded, neoantigen-specific T cells. We propose to categorize neoantigen identification strategies for clinical application into three levels, with level III as the most desirable strategy (Fig. 1).

At level I, mutations are identified by high-throughput DNA/mRNA sequencing of tumor samples and normal tissues. Neoepitopes are algorithmically predicted *in silico* based on a predefined set of criteria. Four clinical trials used the level I neoantigen identification strategy,⁽⁶⁻⁹⁾ although the level III strategy was attempted in one trial.⁽⁹⁾ The advantages of computational *in silico* prediction are the small sample size and short time required for neoantigen prediction. Major drawbacks of the level I neoantigen identification strategy are the uncertainty of whether the predicted

neoepitopes can induce a strong antigen-specific T-cell response in patients (immunogenicity) and whether the neoepitopes are presented by tumor cells (targetability).

The level II clinical neoantigen identification strategy consists of level I identification plus confirmation of neoantigen-specific T-cell clones in patients (Fig. 1). Neoantigen-based adoptive T-cell transfer therapies have identified T-cell clones recognizing neoepitopes from patients' TILs.⁽³⁾ Treatment efficacy suggests that these neoepitopes are presented by MHC molecules on tumor cells, though this was not confirmed. The advantage of level II neoantigen identification is the firm establishment of neoantigen-specific T cells in patients for clinical use. However, the extensive time and cost associated with the level II strategy remain a concern.⁽⁴⁾

The level III neoantigen identification strategy for clinical application consists of level II plus the confirmation of surface-expressed neoepitopes by tumor MHC-I and MHC-II molecules (Fig. 1). The level III strategy has been demonstrated in mouse tumor models⁽¹¹⁾ and has identified neoepitopes in human melanoma samples.⁽¹²⁾ Practical and technical hurdles to implementing the level III strategy to clinical therapy include the requirement for a significant amount of tumor tissue, insensitivity to very low-abundance peptides, and bias toward detecting soluble peptides with high affinity to HLA. Nevertheless, the detection of abundant peptides from TAAs and normal cellular proteins in HLA immunopeptidomes from different tumors indicates the validity of the methodology.

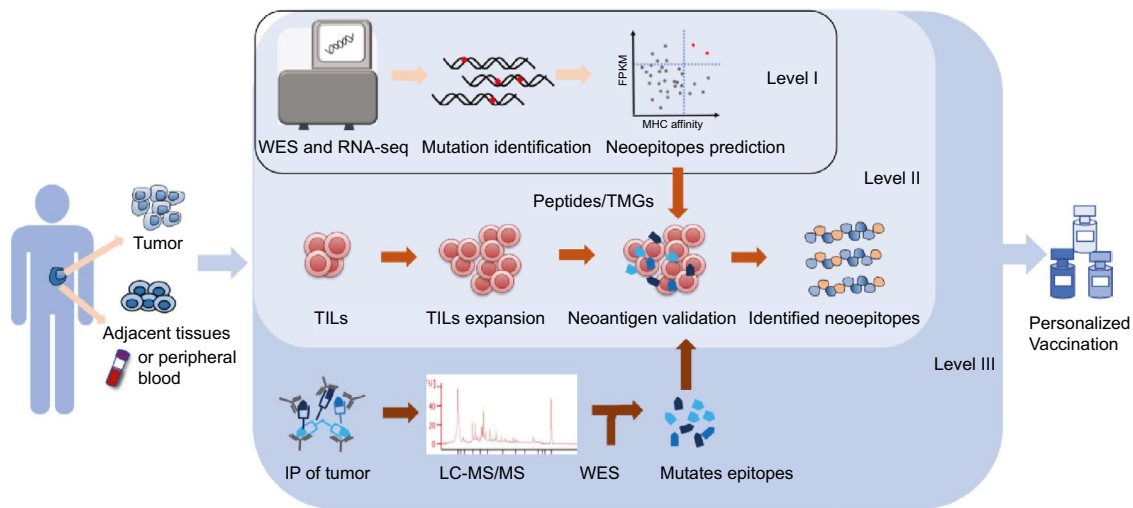


FIG. 1. Three levels of neoantigen identification strategies in clinical application. Level I: patient's tumor tissues and adjacent tissues or peripheral blood are collected for whole-exome and transcriptome sequencing to identify mutations. Neopeptides are predicted by different algorithms based on MHC affinity, location and type of mutations, and mRNA expression abundance. Level II: neopeptides are predicted with level I strategy. Peptides or tandem minigenes are synthesized and cocultured with patient-derived TILs to validate the immunogenicity. Level III: based on the level I prediction, some neopeptides are confirmed in HLA ligandomes presented by patients' tumor MHC-I and MHC-II molecules and then verified for their immunogenicity using patient-derived TILs in level II strategy. The neoantigen identification in HCC⁽¹³⁾ used the level III strategy. Abbreviations: FPKM, fragments per kilobase of transcript per million; IP, immunoprecipitation; LC-MS/MS, liquid chromatography-tandem mass spectrometry; RNA-seq, RNA sequencing; TMG, tandem minigene; WES, whole-exome sequencing.

Quantity and Quality of Neoantigens in HCC

A recent study on the neoantigen landscape in HCC raised concern about the potential pitfalls of targeting neoantigens in this type of tumor.⁽¹³⁾ Of 1,039 nonsynonymous mutations identified in 16 patients with HCC, no neopeptides were detected in HLA class I complexes upon mass spectrometric analysis. In contrast, an average of 1,403 unique peptides derived from TAAs and normal cellular proteins were found in each patient⁽¹³⁾ (Table 1). The median TMB of liver cancer, of which HCC accounts for 90%, is ranked twelfth of 30 different types of cancers,⁽¹⁴⁾ and HCC has a TMB of ~2.0 mutations/megabase, overall an intermediate TMB cancer.^(13,15-18) Importantly, a comparison of HLA-presented neopeptides among five different solid tumor types revealed that the identification of neopeptides in HLA immunopeptidomes is a rare event in tumors with intermediate and low TMBs, like HCC^(9,12,13,19,20) (Table 1). These results clearly demonstrated the low quantity of presented neoantigens in these tumors and suggest that

neoantigen-specific T cells induced by personalized vaccines may not be able to exert cytotoxic killing.

The quality of neoantigens is a critical determinant in tumor rejection and is defined by clonality (uniformly expressed), dissimilarity to self-antigens, mRNA/protein expression levels, HLA binding, and likelihood of neoantigen loss.⁽²¹⁾ The importance of clonal neoantigen expression for effective therapy is supported by clinical and animal studies.^(22,23) However, HCC has poor-quality neoantigens due to high ITH at both the genomic and proteomic levels. ITH in HCC has been extensively studied at multiomics levels and shown to be at a high degree.⁽²⁴⁻³¹⁾ At the genomic level, the number of clonal nonsilent mutations in HCC is ranked eighth among 16 types of cancers, while melanoma is ranked first, indicating that a high TMB does not necessarily translate into high ITH.⁽³²⁾ When the regional distribution of nonsilent mutations was analyzed in patients with HCC, it was found that the percentages of ubiquitous mutations detected in all lesions varied from 8% to 90%⁽²⁷⁾ and that those of heterogeneous mutations varied from 12.9% to 68.5%.⁽³⁰⁾ In addition to genomic ITH as a quality feature, true predicted neoantigens (TPNAs)

should share no homology to self-antigens (dissimilarity). By this criterion, only a handful of TPNA were found *in silico* despite a large number of predicted neoantigens from HCC.⁽³³⁾

The proteomic ITH of neoepitopes in HCC is another major factor impacting on their quality. In two multiomics studies, the majority of the detected somatic mutations in HCC at the DNA and RNA levels were not found at the protein level.^(31,34) Of > 100,000 DNA and RNA variants from 159 patients with HCC, only 1,973 proteomic variants (1.75%) were confirmed by mass spectrometric analysis.⁽³⁴⁾ In a second study on 21 tumor lesions from 8 patients with HCC, of 11,266 and 14,706 nonsynonymous single-nucleotide DNA and RNA variants, respectively, a total of 1,875 single-amino acid variants at the protein level were detected.⁽³¹⁾ Importantly, the proteomic ITH in these patients was extensive as only a few expressed proteins were common to all samples from the same patients.⁽³¹⁾ Taken together, these data suggest that mutated neoantigens in HCC have a high degree of ITH at both the genomic and proteomic levels. Therefore, both the quantity and quality of HCC neoantigens may be limited to serve as effective immunotherapeutic targets.

Strategies to Target Neoantigens in HCC

The rarity of mutated neoantigens in HLA immunopeptidomes from HCC and three other solid tumors is sobering news for clinical trials developing neoantigen-targeted immunotherapies for solid tumors with intermediate/low TMBs. Recent clinical application of neoantigen targeting has largely focused on single-nucleotide variations in coding regions because this type of mutation accounts for the vast majority of cancer mutations.⁽²⁾ Furthermore, most of the analyzed tumor samples are unmanipulated. Thus, two major strategies can be used to broaden the scope of neoantigen targeting. The first strategy is to explore other types of neoantigens derived from largely untapped sources such as frameshift mutations, cryptic noncanonical reading frames, RNA transcription and splicing errors, RNA editing, gene fusion, gene insertions and deletions, intron retentions, noncoding but transcribed and translated regions, posttranslationally

modified glycopeptides, class I-associated phosphopeptides, and nonmutated epitopes presented by the transporter associated with antigen processing (TAP)-independent pathway. Last, but perhaps most important, are the abundantly expressed unmutated epitopes derived from TAAs and normal cellular proteins that are presented by classical antigen presentation pathways.⁽¹⁾ Two clinical studies suggested that using personalized nonmutated epitopes identified by the level III strategy in cancer treatment is effective at controlling disease and prolonging patient survival.^(9,20)

The second strategy involves tumor manipulation to enhance neoantigen generation, expression, and presentation by three potential approaches (Fig. 2). The first approach is to use existing chemotherapeutic/targeted agents and radiotherapy. Chemotherapeutic agents kill tumor cells by causing DNA damage and enhancing tumor mutations. Treatment of tumor cells with epigenetic modulators such as histone deacetylase inhibitors results in increased expression of tumor antigens.⁽³⁵⁾ Radiotherapy enhances neoantigen expression.⁽³⁶⁾ Additionally, targeted agents against epidermal growth factor receptor/B-Raf proto-oncogene mutations down-regulated mismatch repair (MMR) and enhanced colorectal cancer mutation.⁽³⁷⁾ These studies suggest that existing treatments are useful means to enhance neoantigen generation and expression for combination therapy.

The second approach is to use oncolytic viruses. Viral oncolysis of tumors plus anti-programmed cell death 1 (PD-1) significantly broadened the spectrum of CD8⁺ T-cell responses to neoepitopes and controlled disease better than anti-PD-1 monotherapy.⁽³⁸⁾ The efficacy of oncolytic virus therapy is likely due to both enhanced cross-presentation of neoantigens by host dendritic cells and enhanced expression and presentation of neoepitopes on tumor cells.⁽³⁹⁾ Given the rapid development of oncolytic viruses as cancer drugs, an oncolytic virus-mediated approach to enhance neoantigen expression and presentation is feasible and may become an important strategy in cancer therapy.

The third approach is to disrupt endogenous pathways in tumor cells by genetic means. Patients with tumor cells deficient in the DNA MMR pathway have high numbers of somatic mutations and are extraordinarily sensitive to immune checkpoint blockade-based therapy.⁽⁴⁰⁾ Supporting these observations, clustered regularly interspaced short palindromic repeats (CRISPR)/

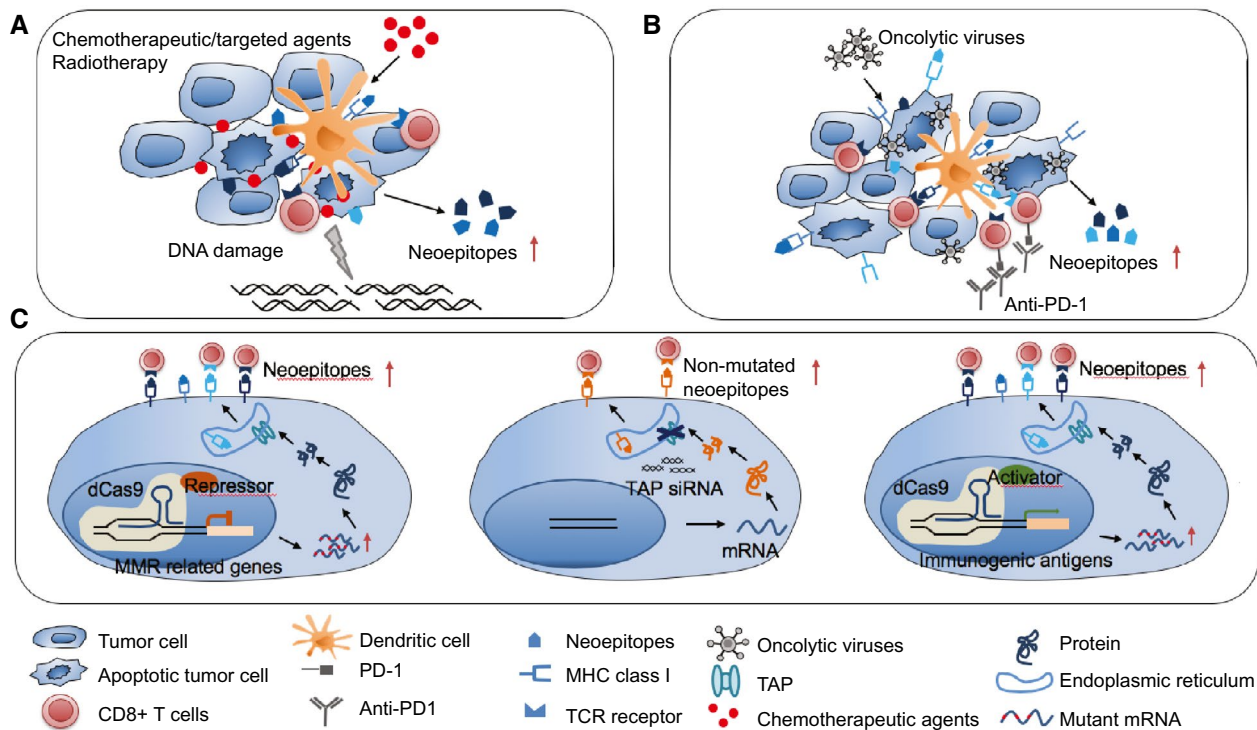


FIG. 2. Strategies to enhance neoantigen generation, expression, and presentation in HCC and other solid tumors. (A) Chemotherapeutic/targeted agents and radiotherapy kill tumor cells by causing DNA damage and enhance neoantigen expression. (B) Oncolytic virus infection can increase expression and presentation of neoepitopes on tumor cells and enhance cross-presentation of neoantigens by host dendritic cells. Viral oncolysis of tumors plus anti-PD-1 broaden the spectrum of CD8⁺ T-cell responses to neoepitopes. (C) Genetic means to disrupt endogenous pathways in tumor cells to increase neoepitope release: inactivation of the MMR pathway through CRISPR-Cas9-mediated gene editing; TAP down-regulation through TAP small interfering RNA conjugated to tumor-targeting nucleolin aptamer; activation of endogenous genes in tumors by CRISPR activation-based technology. Abbreviations: siRNA, small interfering RNA; TCR, T-cell receptor.

CRISPR-associated 9 (Cas9)-mediated disruption of MMR genes resulted in increased generation of neoantigens and improved tumor control through immune surveillance.⁽⁴¹⁾ TAP-deficient cells can present non-mutated neoepitopes,⁽⁴²⁾ and silencing TAP expression in tumor cells inhibited tumor growth in mice and enhanced the antitumor-specific T-cell response.⁽⁴³⁾ Recent studies demonstrate that CRISPRa-based technologies augment both antigen presentation and antitumor T-cell priming.⁽⁴⁴⁾ Although promising in animal studies, these approaches still need tremendous technical advancement for human application.

In summary, although neoantigens are promising targets in cancer therapy, application of this class of antigens for immunotherapy of HCC and other tumors with intermediate and low TMBs faces major challenges. The major limitation in these cancers is the lack of targetability due to rare presentation by

HLA complexes on tumor cells. Thus, strategies are needed to enhance the generation, expression, and presentation of neoantigens in tumors. The three approaches outlined herein will likely enhance the generation, expression, and presentation of TAAs and neoantigens and warrant further clinical investigation.

Author Contributions: L.L. and Y-W.H. were responsible for the conception. Y-W. H., J.J. and H.Z. were drafting and revising the paper. M.Z., Q-T.W., S-N.S., X-K.G., H.Y., Y.W., J.O.L., S-Y.L., and Y.L. contributed to conception, discussion and review of the paper.

REFERENCES

- 1) Finn OJ, Rammensee HG. Is it possible to develop cancer vaccines to neoantigens, what are the major challenges, and how can these be overcome? Neoantigens: nothing new in spite of the name. *Cold Spring Harb Perspect Biol* 2018;10:a028829.

- 2) Vormehr M, Tureci O, Sahin U. Harnessing tumor mutations for truly individualized cancer vaccines. *Annu Rev Med* 2019;70:395-407.
- 3) Tran E, Turcotte S, Gros A, Robbins PF, Lu YC, Dudley ME, et al. Cancer immunotherapy based on mutation-specific CD4⁺ T cells in a patient with epithelial cancer. *Science* 2014;344:641-645.
- 4) Yamamoto TN, Kishton RJ, Restifo NP. Developing neoantigen-targeted T cell-based treatments for solid tumors. *Nat Med* 2019;25:1488-1499.
- 5) Carreno BM, Magrini V, Becker-Hapak M, Kaabinejadian S, Hundal J, Petti AA, et al. Cancer immunotherapy: A dendritic cell vaccine increases the breadth and diversity of melanoma neoantigen-specific T cells. *Science* 2015;348:803-808.
- 6) Ott PA, Hu Z, Keskin DB, Shukla SA, Sun J, Bozym DJ, et al. An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature* 2017;547:217-221.
- 7) Sahin U, Derhovanesian E, Miller M, Kloke BP, Simon P, Lower M, et al. Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature* 2017;547:222-226.
- 8) Keskin DB, Anandappa AJ, Sun J, Tirosch I, Mathewson ND, Li S, et al. Neoantigen vaccine generates intratumoral T cell responses in phase Ib glioblastoma trial. *Nature* 2019;565:234-239.
- 9) Hilf N, Kuttruff-Coqui S, Frenzel K, Bukur V, Stevanovic S, Gouttefangeas C, et al. Actively personalized vaccination trial for newly diagnosed glioblastoma. *Nature* 2019;565:240-245.
- 10) Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 2005;352:987-996.
- 11) Yadav M, Jhunjhunwala S, Phung QT, Lupardus P, Tanguay J, Bumbaca S, et al. Predicting immunogenic tumour mutations by combining mass spectrometry and exome sequencing. *Nature* 2014;515:572-576.
- 12) Bassani-Sternberg M, Braunlein E, Klar R, Engleitner T, Sinitcyn P, Audehm S, et al. Direct identification of clinically relevant neoepitopes presented on native human melanoma tissue by mass spectrometry. *Nat Commun* 2016;7:13404.
- 13) Loffler MW, Mohr C, Bichmann L, Freudenmann LK, Walzer M, Schroeder CM, et al. Multi-omics discovery of exome-derived neoantigens in hepatocellular carcinoma. *Genome Med* 2019;11:28.
- 14) Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, et al. Signatures of mutational processes in human cancer. *Nature* 2013;500:415-421.
- 15) Ahn SM, Jang SJ, Shim JH, Kim D, Hong SM, Sung CO, et al. Genomic portrait of resectable hepatocellular carcinomas: implications of RB1 and FGF19 aberrations for patient stratification. *HEPATOLOGY* 2014;60:1972-1982.
- 16) Huang J, Deng Q, Wang Q, Li KY, Dai JH, Li N, et al. Exome sequencing of hepatitis B virus-associated hepatocellular carcinoma. *Nat Genet* 2012;44:1117-1121.
- 17) Schulze K, Imbeaud S, Letouze E, Alexandrov LB, Calderaro J, Rebouissou S, et al. Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets. *Nat Genet* 2015;47:505-511.
- 18) Fujimoto A, Furuta M, Totoki Y, Tsunoda T, Kato M, Shiraishi Y, et al. Whole-genome mutational landscape and characterization of noncoding and structural mutations in liver cancer. *Nat Genet* 2016;48:500-509.
- 19) Newey A, Griffiths B, Michaux J, Pak HS, Stevenson BJ, Woolston A, et al. Immunopeptidomics of colorectal cancer organoids reveals a sparse HLA class I neoantigen landscape and no increase in neoantigens with interferon or MEK-inhibitor treatment. *J Immunother Cancer* 2019;7:309.
- 20) Loffler MW, Chandran PA, Laske K, Schroeder C, Bonzheim I, Walzer M, et al. Personalized peptide vaccine-induced immune response associated with long-term survival of a metastatic cholangiocarcinoma patient. *J Hepatol* 2016;65:849-855.
- 21) McGranahan N, Swanton C. Neoantigen quality, not quantity. *Sci Transl Med* 2019;11:eaax7918.
- 22) McGranahan N, Furness AJ, Rosenthal R, Ramskov S, Lyngaa R, Saini SK, et al. Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. *Science* 2016;351:1463-1469.
- 23) Gejman RS, Chang AY, Jones HF, DiKun K, Hakimi AA, Schietinger A, et al. Rejection of immunogenic tumor clones is limited by clonal fraction. *eLife* 2018;7:e41090.
- 24) Ling S, Hu Z, Yang Z, Yang F, Li Y, Lin P, et al. Extremely high genetic diversity in a single tumor points to prevalence of non-Darwinian cell evolution. *Proc Natl Acad Sci U S A* 2015;112:E6496-E6505.
- 25) Shi JY, Xing Q, Duan M, Wang ZC, Yang LX, Zhao YJ, et al. Inferring the progression of multifocal liver cancer from spatial and temporal genomic heterogeneity. *Oncotarget* 2016;7:2867-2877.
- 26) Hou Y, Guo H, Cao C, Li X, Hu B, Zhu P, et al. Single-cell triple omics sequencing reveals genetic, epigenetic, and transcriptomic heterogeneity in hepatocellular carcinomas. *Cell Res* 2016;26:304-319.
- 27) Xue R, Li R, Guo H, Guo L, Su Z, Ni X, et al. Variable intra-tumor genomic heterogeneity of multiple lesions in patients with hepatocellular carcinoma. *Gastroenterology* 2016;150:998-1008.
- 28) Zhai W, Lim TK, Zhang T, Phang ST, Tiang Z, Guan P, et al. The spatial organization of intra-tumour heterogeneity and evolutionary trajectories of metastases in hepatocellular carcinoma. *Nat Commun* 2017;8:4565.
- 29) Lin DC, Mayakonda A, Dinh HQ, Huang P, Lin L, Liu X, et al. Genomic and epigenomic heterogeneity of hepatocellular carcinoma. *Cancer Res* 2017;77:2255-2265.
- 30) Gao Q, Wang ZC, Duan M, Lin YH, Zhou XY, Worthley DL, et al. Cell culture system for analysis of genetic heterogeneity within hepatocellular carcinomas and response to pharmacologic agents. *Gastroenterology* 2017;152:232-242.e234.
- 31) Zhang Q, Lou Y, Yang J, Wang J, Feng J, Zhao Y, et al. Integrated multiomic analysis reveals comprehensive tumour heterogeneity and novel immunophenotypic classification in hepatocellular carcinomas. *Gut* 2019;68:2019-2031.
- 32) McGranahan N, Swanton C. Clonal heterogeneity and tumor evolution: past, present, and the future. *Cell* 2017;168:613-628.
- 33) Petrizzo A, Tagliamonte M, Mauriello A, Costa V, Aprile M, Esposito R, et al. Unique true predicted neoantigens (TPNAs) correlates with anti-tumor immune control in HCC patients. *J Transl Med* 2018;16:286.
- 34) Gao Q, Zhu H, Dong L, Shi W, Chen R, Song Z, et al. Integrated proteogenomic characterization of HBV-related hepatocellular carcinoma. *Cell* 2019;179:561-577.e522.
- 35) van den Bijgaart RJE, Kroesen M, Wassink M, Brok IC, Kers-Rebel ED, Boon L, et al. Combined sialic acid and histone deacetylase (HDAC) inhibitor treatment up-regulates the neuroblastoma antigen GD2. *J Biol Chem* 2019;294:4437-4449.
- 36) Formenti SC, Rudqvist NP, Golden E, Cooper B, Wennerberg E, Lhuillier C, et al. Radiotherapy induces responses of lung cancer to CTLA-4 blockade. *Nat Med* 2018;24:1845-1851.
- 37) Russo M, Crisafulli G, Sogari A, Reilly NM, Arena S, Lamba S, et al. Adaptive mutability of colorectal cancers in response to targeted therapies. *Science* 2019;366:1473-1480.
- 38) Woller N, Gurlevik E, Fleischmann-Mundt B, Schumacher A, Knoke S, Kloos AM, et al. Viral Infection of tumors overcomes resistance to PD-1-immunotherapy by broadening neoantigenome-directed T-cell responses. *Mol Ther* 2015;23:1630-1640.
- 39) Murphy JP, Kim Y, Clements DR, Konda P, Schuster H, Kowalewski DJ, et al. Therapy-induced MHC I ligands shape

neo-antitumor CD8 T cell responses during oncolytic virus-based cancer immunotherapy. *J Proteome Res* 2019;18:2666-2675.

- 40) Le DT, Durham JN, Smith KN, Wang H, Bartlett BR, Aulakh LK, et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science* 2017;357:409-413.
- 41) Germano G, Lamba S, Rospo G, Barault L, Magri A, Maione F, et al. Inactivation of DNA repair triggers neoantigen generation and impairs tumour growth. *Nature* 2017;552:116-120.
- 42) Marijt KA, Blijleven L, Verdegaal EME, Kester MG, Kowalewski DJ, Rammensee HG, et al. Identification of non-mutated neoantigens presented by TAP-deficient tumors. *J Exp Med* 2018;215:2325-2337.
- 43) Garrido G, Schrand B, Rabasa A, Levay A, D'Eramo F, Berezhnoy A, et al. Tumor-targeted silencing of the peptide transporter TAP induces potent antitumor immunity. *Nat Commun* 2019; 10:3773.
- 44) Wang G, Chow RD, Bai Z, Zhu L, Errami Y, Dai X, et al. Multiplexed activation of endogenous genes by CRISPRa elicits potent antitumor immunity. *Nat Immunol* 2019;20: 1494-1505.
- 45) Chaisaingmongkol J, Budhu A, Dang H, Rabibhadana S, Papatdi B, Kwon SM, et al. Common molecular subtypes among asian hepatocellular carcinoma and cholangiocarcinoma. *Cancer Cell* 2017;32:57-70.e53.