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Safety and Clinical Benefit of Personalized Neoantigen Vaccines in Preventing Postoperative Recurrence of High-Risk Hepatocellular Carcinoma

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ABSTRACT

Clinically, there are very few prophylactic therapies available to prevent recurrence in patients with hepatocellular carcinoma (HCC) following curative resection. Although neoantigen-based vaccines have shown the capacity to provoke strong antitumor immunity across multiple solid malignancies, their ability to trigger such immunity in HCC—and to act as a safe, efficacious prophylactic measure against postoperative recurrence—remains poorly understood. A customized neoantigen vaccine was developed and delivered to 10 high-risk HCC patients in a prime-boost regimen. Safety and immunological reactivity were monitored through adverse event recording, tissue sequencing, ELISpot assays, and T-cell receptor sequencing. Clinical outcomes were measured by recurrence-free survival (RFS) and patient-specific circulating tumor DNA (ctDNA) analysis. None of the 10 patients experienced notable adverse effects during vaccination. By the trial's data cutoff, imaging confirmed recurrence in 8 patients, whereas 2 remained free of relapse. The median RFS from the initial vaccination across all 10 patients was 7.4 months. Among the 7 patients who received the full vaccination schedule, 5 displayed detectable neoantigen-specific T-cell responses and achieved markedly prolonged RFS after surgery compared with the remaining 5 patients (lacking responsive neoantigens or receiving only the prime dose) and propensity score-matched controls ($p = 0.035$). Additionally, serial detection of patient-specific neoantigen mutations in ctDNA allowed real-time monitoring of treatment response and disease status during vaccination and subsequent follow-up. This study establishes personalized neoantigen vaccination as a safe, practical, and promising approach for preventing HCC recurrence. Disease dynamics can be accurately tracked via neoantigen mutation signatures in ctDNA, offering valuable insights for tailored management of HCC patients.

Keywords: Neoantigen vaccine, HCC, Anti-recurrence, Prophylactic treatmen, Circulating tumor DNA

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Introduction

Hepatocellular carcinoma (HCC) poses a worsening global public health challenge. In China alone, new cases represent 55% of the global burden, with around 422,100 annual deaths attributable to disease advancement [1]. Many patients are diagnosed with vascular invasion of varying severity, which contributes to low resectability rates and dismal outcomes. Nonetheless, evidence from earlier trials indicates that select patients with portal vein branch involvement can still gain survival advantage from resection [2, 3], aligning with recommendations in the Chinese Guidelines for Diagnosis and Treatment of Primary Liver Cancer (2019 Edition) and EASL Clinical Practice Guidelines [4, 5]. Despite surgical intervention, these individuals face a substantial likelihood of recurrence and distant spread. Effective postoperative prophylactic options remain scarce. In China, Transcatheter Arterial Chemoembolization (TACE) is commonly employed for prevention, yet it lacks strong evidentiary support and is not endorsed by EASL or NCCN guidelines. Thus, identifying innovative and reliable methods to reduce postoperative recurrence is critically needed.

Neoantigens are immunogenic peptides arising exclusively from tumor-specific nonsynonymous alterations. Presented on tumor cells via major histocompatibility complex (MHC) molecules, they enable precise T-cell

recognition and potent antitumor immunity. Unlike vaccines targeting tumor-associated antigens, neoantigen-directed vaccines offer enhanced therapeutic potential with reduced risk of autoimmune complications, given their tumor-exclusive expression [6]. These antigens commonly stem from point mutations in DNA, RNA editing, indel events, gene fusions, and related genomic changes [7]. Recent clinical investigations have confirmed that neoantigens from point mutations can drive vigorous antitumor responses in melanoma, small cell lung cancer, and glioma patients [8–10]. In contrast, the immunogenicity of neoantigen vaccines in HCC—a malignancy with moderate tumor mutation burden of approximately 2.0 mutations per megabase [11, 12]—is uncertain. Prior work has linked higher neoantigen loads in HCC to tumor evolutionary patterns and survival outcomes, supporting their potential as prime targets for immunotherapy [13, 14]. Furthermore, RNA editing has emerged as an additional source of neoantigens [15], expanding options for tumors with limited mutational loads. Whether these alternative sources can elicit meaningful antitumor immunity in HCC warrants further examination.

Furthermore, obtaining timely and reliable monitoring of therapeutic response and patient outcomes continues to pose a major difficulty in HCC follow-up. Because of their inadequate sensitivity and specificity, conventional imaging (CT/MRI) and serum protein markers are unable to offer immediate, dynamic insights into tumor load, particularly for detecting minimal residual disease (MRD) after curative resection. Prior work from our group showed that circulating tumor DNA (ctDNA)—brief DNA segments shed by tumor cells into the bloodstream—can act as a noninvasive, highly responsive biomarker for real-time surveillance of HCC tumor burden via detection of individual-specific tumor mutations [16]. Similarly, Jia *et al*. found that following patient-unique neoantigen loci in ctDNA precisely captured treatment responses during immune checkpoint inhibition in non-small cell lung cancer [17]. Accordingly, ctDNA holds promise as a precise and sensitive method for tracking clinical outcomes in the context of neoantigen-directed immunotherapy and deserves comprehensive study.

In this prospective trial, we included 10 individuals with surgically removable HCC accompanied by portal vein branch vascular invasion, a factor linked to heightened postoperative recurrence probability. Upon identifying candidate neoantigens unique to each case, we thoroughly investigated whether a customized long-peptide neoantigen vaccine could provide a tolerable and potent means of reducing recurrence risk after resection. We also constructed bespoke somatic mutation panels encompassing neoantigen-related sites to facilitate ctDNA sequencing and enable ongoing evaluation of treatment effects during the immunotherapy phase.

Materials and Methods

Clinical trial design and treatment

This was an investigator-sponsored, single-arm, open-label trial performed at Mengchao Hepatobiliary Hospital of Fujian Medical University in China, registered on the Chinese Clinical Trial Registry (http://www.chictr.org.cn/; ChiCTR1900020990). The main goal was to determine the tolerability and practicality of using a tailored neoantigen vaccine to reduce HCC recurrence post-hepatectomy. The trial design, protocol, and modifications were approved by the Institutional Review Board at Mengchao Hepatobiliary Hospital of Fujian Medical University (Fujian, China). Every procedure and specimen collection complied with the Declaration of Helsinki and International Conference on Harmonization Good Clinical Practice guidelines. All participants provided signed informed consent. Primary outcomes centered on vaccine tolerability and practicality, with secondary outcomes encompassing immunological reactivity and recurrence-free survival (RFS). Principal enrollment requirements included: (1) men or women aged 18 to 75 years, with serum bilirubin $\leq 1.5\times$ upper limit of normal (ULN) and ALT/AST $\leq 2.0\times$ ULN; (2) verified resectable HCC or intrahepatic cholangiocarcinoma (CC) absent distant spread; (3) tumor thrombus in a portal vein branch confirmed histologically or visibly during operation. Primary exclusion factors were: (1) presence of HIV/HCV, significant coronary disease, or other investigator-judged contraindications; (2) previous bone marrow or solid organ transplant; (3) any immunodeficiency or autoimmune disorder history; (4) immunotherapy exposure within 1 month prior or fewer than five suitable neoepitopes identified.

Once diagnosed, participants first underwent complete surgical removal of the tumor, followed by adjuvant TACE within 2 months. Customized neoantigen vaccines were subsequently prepared and given subcutaneously following a prime-boost protocol. Patients with radiologically verified recurrence ended trial participation and proceeded to routine care. Tolerability after every dose was monitored via physical exams, standard hematology/biochemistry, and ECG.

In parallel, 20 comparable HCC cases untreated with neoantigen vaccine were chosen as controls through propensity score matching (PSM). These controls shared key baseline features with the treated cohort, including therapeutic approach (laparoscopic surgery plus prophylactic TACE), gender, age, vascular involvement, hemoglobin (≥100 g/dl), and platelets (≥80/l). Matching utilized a 1:2 nearest-neighbor method with a 0.2 caliper. Tumor specimens and surrounding nontumor liver tissue were harvested during resection. Sequential blood draws for plasma and peripheral blood mononuclear cells (PBMCs) occurred across the study timeline—pre-surgery, post-surgery, during vaccination, and at follow-up visits. Assessments of treatment effect (imaging plus ctDNA analysis) and immune activation (tissue sequencing, immunohistochemistry, ELISpot, TCR profiling, T-cell subset flow cytometry, and cytokine measurements) took place throughout vaccination and surveillance phases.

Tissue sequencing and epitope prediction

Candidate neoantigens were identified through whole-exome sequencing (200× depth) and RNA sequencing of paired HCC tumor and adjacent normal liver samples. In short, DNA and RNA were isolated from each patient's matched tissues, and sequencing libraries were prepared following standard protocols before being sequenced by Fulgent Co., Ltd. on the Illumina NovaSeq 6000 system (paired-end, 150 bp).

Four-digit resolution typing for HLA class I loci (HLA-A, HLA-B, HLA-C) was performed with OptiType [18] using RNA-seq reads, while HLA class II (HLA-DRB1) typing employed Seq2HLA. Exome data were analyzed for somatic variants with MuTect2 and SomaticSniper; overlapping calls were refined using DeepSNV. Variants were then cross-checked against RNA-seq, retaining only those achieving ≥ 20× coverage and VAF ≥ 0.05 in tumor transcripts. Potential immunogenicity was scored via the pVAC-Seq pipeline [19]. Missense variants generated mutant peptides (8–11 mers for class I; 15 mers for class II), with binding strengths estimated by NetMHCpan, NetMHC, NetMHCcons, PickPocket, and MHCflurry (class I) or NetMHCIIpan (class II). Variants producing peptides with median IC50 < 500 nM to either class I or class II HLA were classified as candidate neoantigens. If ≤ 30 candidates emerged, all advanced to 27-mer peptide synthesis. For > 30 candidates, prioritization followed this sequence: (1) strong binders (IC50 < 150 nM) to both class I and class II; (2) strong binders to one class; (3) elevated RNA VAF; (4) intermediate binders (150 < IC50 ≤ 500 nM) to either class.

Personalized neoantigen long-peptide vaccine synthesis and vaccination

Up to 30 top-ranked neoantigen candidates per patient were chosen for production of 27-amino-acid clinical-grade peptides via solid-phase synthesis under GMP-equivalent standards (> 98% purity; endotoxin < 0.01 EU/g). In practice, 6–20 peptides per patient were manufactured within the required timeline. Peptides were organized into 2–4 pools (pools 1–4; 3–5 peptides per pool at 0.3 mg each) and passed comprehensive quality checks for sterility, pyrogens, and toxicity. Each pool was formulated with 0.5 mg poly-IC adjuvant (polyinosinic-polycytidylic acid; Guangdong South China Pharmaceutical Co.) and injected subcutaneously in rotating bilateral axillary and inguinal regions. The regimen included prime injections on days 1, 4, 8, 15, and 22, with booster injections on days 90 and 140 (allowing a ±15-day flexibility for boosters).

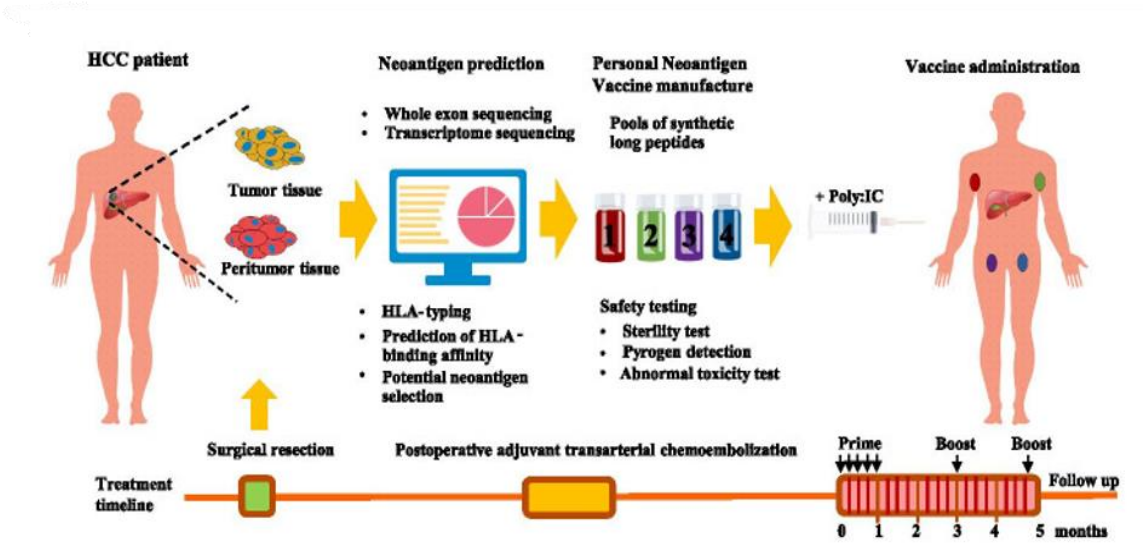
Statistical analysis

All patients completing at least 5 prime-phase doses were incorporated into safety and efficacy analyses. Safety data were presented descriptively. Graphs for recurrence-free survival (RFS), immunological reactivity, and ctDNA trends were created using GraphPad Prism 6 or R software.

Results and Discussion

Study design and patient characteristics

This single-arm prospective trial began with bespoke neoantigen discovery for each enrolled HCC patient exhibiting vascular invasion, leveraging whole-exome and transcriptome data from resected tumor and paired peritumor specimens **(Figure 1a).** Individualized vaccines comprised 6–20 synthetic 27-mer peptides (sourced from point mutations or RNA editing events), assembled into 1–4 pools (pools 1–4; each holding 3–5 peptides). After passing quality assurance, vaccines were co-administered with poly-IC adjuvant via subcutaneous injection to alternating axillary and inguinal sites on a prime-boost timetable (prime: days 1, 4, 8, 15, 22; boost: days 90, 140) subsequent to curative hepatectomy and adjuvant TACE.



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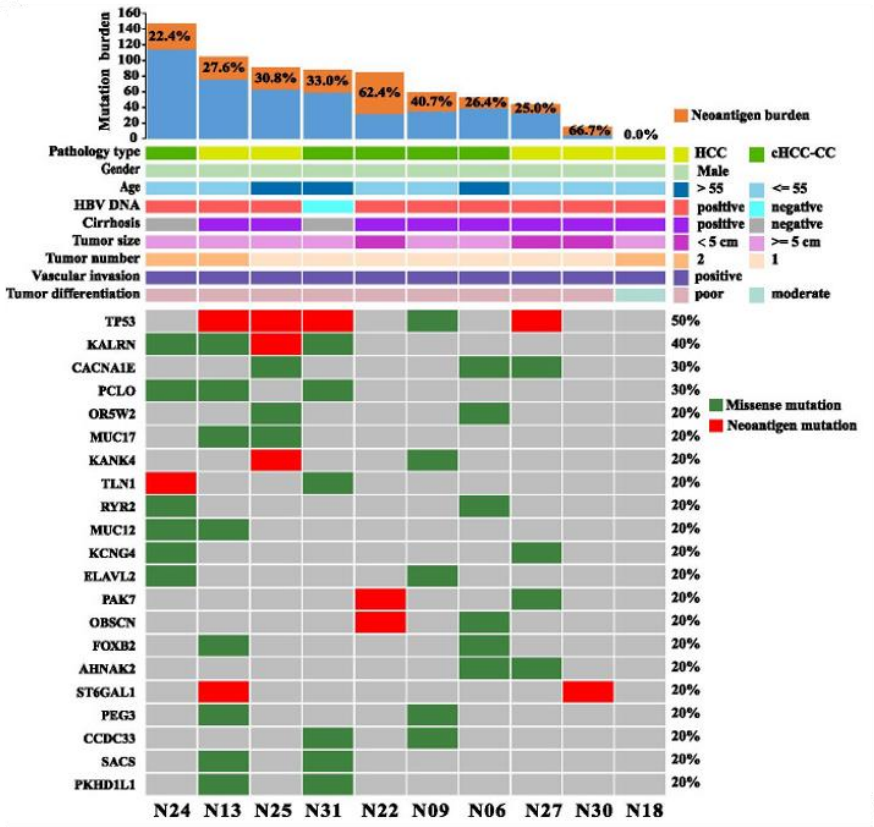

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Figure 1. The overview of study design, patient characteristics, and neoantigen profiles. a) The administration schedule of customized neoantigen vaccine in HCC patients presenting with vascular invasion. b) Quantity of somatic mutations and predicted neoantigens identified in tumor tissue from each patient, along with relevant clinicopathologic details for the 10 participants. The indicated percentage represents the ratio of neoantigen mutations to total somatic mutations. A heatmap displays the distribution of frequently mutated somatic/neoantigenic genes across the cohort. Notably, all neoantigen mutations in patients N06 and N09 originated from patient-specific alterations rather than common hotspot genes.

Based on investigator evaluation, 10 individuals with resectable HCC were recruited to assess the safety and effectiveness of neoantigen vaccination. As illustrated in **Figure 1b,** pathologic examination verified pure HCC in 5 patients, while the remaining 5 were diagnosed with combined hepatocellular carcinoma and intrahepatic

cholangiocarcinoma (cHCC-CC). Nine patients had evidence of HBV infection, and 8 presented with concurrent liver cirrhosis. The mean tumor size across the 10 patients was 7.7 cm (range, 2.8~11 cm).

A total of 780 nonsynonymous somatic single-nucleotide variants (SNVs) were detected, averaging 78 per patient (range, 7~148), through whole-exome sequencing of tumor and matched peritumor samples **(Figure 1b).** Of these, 33.5% (range, 0~66.7%) were expressed at the transcript level (variant allele frequency ≥ 0.05) and simultaneously qualified as neoantigens based on predicted binding affinity (IC50 < 500 nM to HLA class I or II).

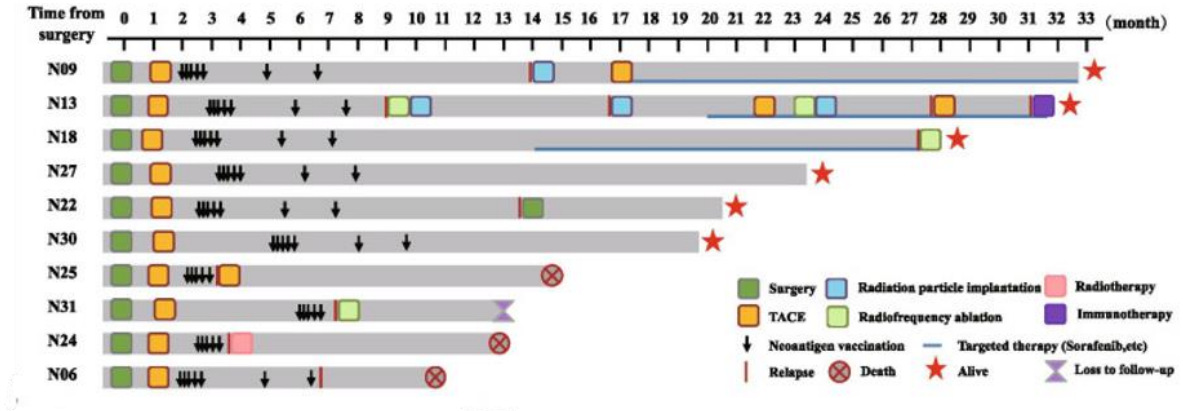
As depicted in **Figure 1b,** recurrent missense mutations in coding regions of genes such as TP53 and KALRN occurred in 50% and 40% of patients, respectively. Consistent with this, neoantigens arising from TP53 alterations were found in 40% (4/10) of cases. Remarkably, patient N18 exhibited no nonsynonymous somatic SNVs at the DNA level. However, 20 tumor-exclusive nonsynonymous RNA editing events were discovered, of which 6 met neoantigen criteria. Existing evidence supports the potential immunogenicity of neoantigens derived from RNA editing [15]. Following thorough discussion of risks and benefits and acquisition of informed consent, a vaccine incorporating these RNA editing-based neoantigens was manufactured and administered to patient N18 in a prime-boost regimen.

The safety of neoantigen vaccination

The median interval between hepatectomy and initial vaccination was 86 days (range, 59 to 159 days). All 10 participants completed the 5 scheduled prime-phase doses, and 7 also received the 2 planned boost doses. No significant treatment-associated adverse events emerged, and serial blood and biochemical parameters remained unremarkable throughout the vaccination period. Only mild (Grade 1) reactions, including local injection-site effects and transient fatigue, were noted; these resolved spontaneously without intervention. These findings confirm the favorable safety profile of neoantigen vaccines.

Clinical outcome and immune response monitoring during vaccination and follow up

A comprehensive timeline of treatments, vaccination schedules, and clinical events is presented in **Figure 2a.** From November 26, 2018, to June 30, 2021, the median follow-up duration for the 10 patients was 20.1 months (range, 10.9~32.7 months). Imaging (MRI/CT) verified recurrence in 8 patients, yielding a median recurrence-free survival (RFS) of 8.3 months post-resection; the remaining 2 patients showed no evidence of relapse (mean follow-up 21.4 months). Kaplan-Meier analysis revealed a median RFS of 7.4 months from the first neoantigen vaccination **(Figure 2b).**



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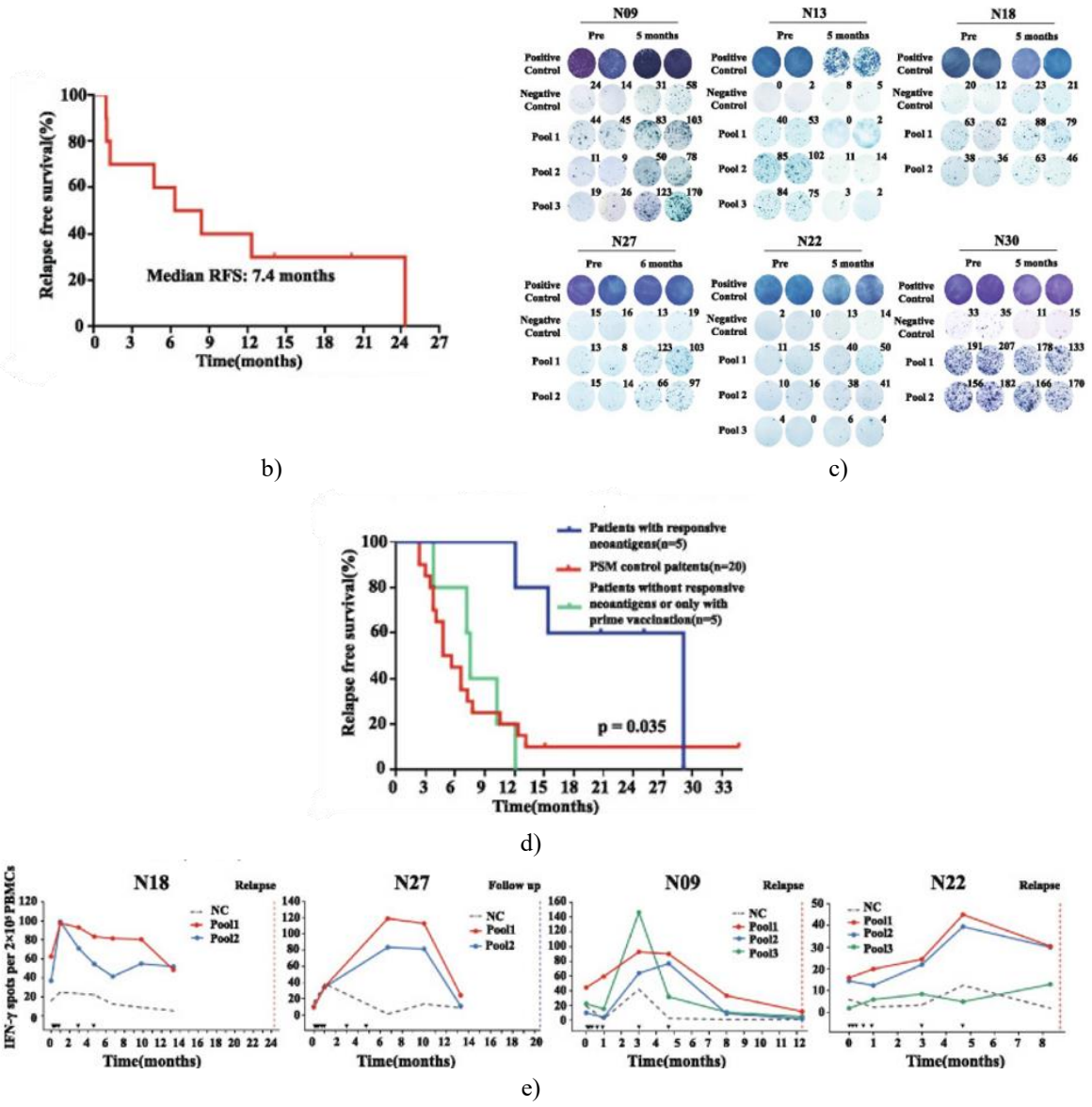


b) c) d) e)

Figure 2. Clinical outcome and immune response monitoring in enrolled HCC patients during vaccination and follow up. a) Detailed chronological overview of surgical interventions, vaccinations, and clinical progression for the 10 patients from resection through trial cutoff. b) Kaplan-Meier plot of RFS calculated from the first neoantigen vaccination across all 10 participants. c) Ex vivo IFN-γ ELISpot results from PBMCs stimulated with patient-specific neoantigen pools, comparing pre- and post-vaccination responses in 6 individuals. d) Kaplan-Meier RFS curves after radical surgery for patients exhibiting responsive neoantigens versus those lacking responses or receiving only prime doses, alongside propensity score-matched controls. e) Longitudinal Ex vivo IFN-γ ELISpot tracking of responses to personalized neoantigen pools in 4 patients. PC denotes positive control; NC denotes negative control.

Generally, neoantigen vaccines require completion of both prime and boost phases, typically taking 2~5 months to establish a strong neoantigen-specific antitumor immune response in the body. However, 3 patients (N24, N25, and N31) experienced recurrence within 2 weeks following the prime phase and therefore did not receive the boost doses **(Figure 2a),** potentially allowing inadequate time for sufficient immune activation. Consequently, ex vivo IFN-γ ELISpot assays were conducted to assess immune responses in the remaining 7 patients who completed the full vaccination schedule, using autologous PBMCs stimulated with neoantigen pool-pulsed autologous dendritic cells. Patient N06 showed no evident IFN-γ response throughout the vaccination period. In contrast, 3 patients (N09, N22, and N27) developed substantial neoantigen-specific PBMC reactivity against the corresponding vaccine peptide pools post-vaccination compared to negative controls and pre-vaccination baselines **(Figure 2c).** Additionally, baseline neoantigen peptide pool reactivity was detected in 3 patients (N13, N18, and N30) prior to

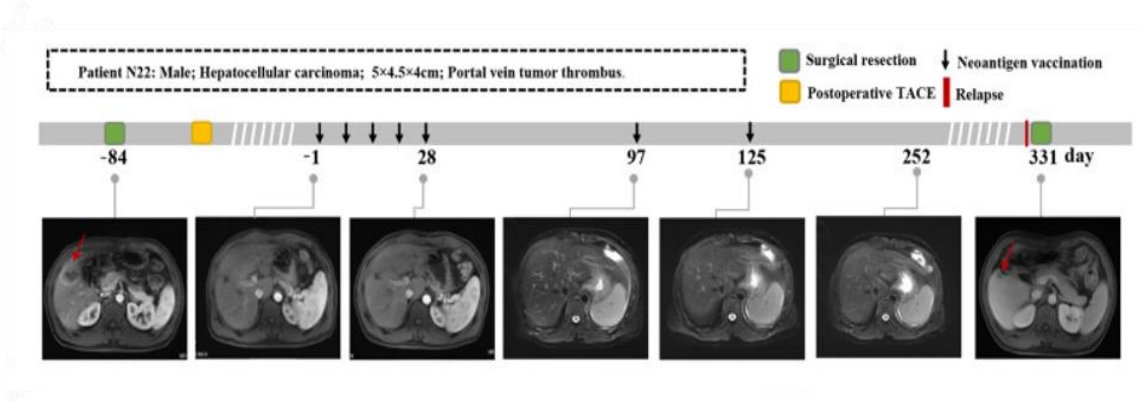
vaccination, possibly resulting from the release of tumor neoantigens from minimal residual disease triggered by prophylactic TACE, thereby priming specific T cells. Notably, in patient N13, this initial response declined sharply during vaccination and became undetectable after the boost phase, indicating failure to sustain or enhance neoantigen-specific T-cell activation. Conversely, patient N18 exhibited markedly stronger post-vaccination reactivity than at baseline, while patient N30 retained comparable response levels, likely supported by the combined effects of vaccination and TACE or inherent immune sensitivity to neoantigens. Further individual peptide testing confirmed that 36 out of 51 (70.6%) long peptides elicited significant specific immune responses in 5 patients (N09, N18, N22, N27, and N30), whereas no responsive peptides were observed in the other 2 patients (N06 and N13) after full vaccination. Of particular interest, all 6 neoantigen peptides derived from RNA editing events in patient N18 achieved a 100% response rate, highlighting the viability of RNA editing sites as neoantigen sources in tumors with low mutation burden. Thus, 5 patients (N09, N18, N22, N27, and N30) were classified as having responsive neoantigens post-vaccination. Integrating clinical outcomes revealed that these responders achieved significantly extended recurrence-free survival (RFS) after curative resection compared to non-responders, patients receiving only prime doses, and propensity score-matched controls (median RFS: 19.3 vs 6.7 vs 4.8 months, P = 0.035), **(Figure 2d).** In parallel, changes in peripheral blood T-lymphocyte subset proportions and serum concentrations of 6 cytokines (IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ) were monitored across all 10 patients during vaccination, yet no meaningful associations emerged with neoantigen responsiveness or RFS (data not shown). Collectively, these findings suggest that vaccine-induced IFN-γ responses serve as a reliable marker of neoantigen vaccine efficacy.

Subsequently, the persistence of neoantigen-specific immunity was examined in 4 patients (N09, N18, N22, and N27) with available follow-up PBMC samples. As illustrated in **Figure 2e**, robust responses persisted up to 10 months post-vaccination in 2 patients (N18: mean 67.8 spots across 2 pools; N27: mean 97.3 spots across 2 pools), whereas responses had weakened by 8 months in patient N09 (mean 17.8 spots across 3 pools) and patient N22 (mean 24.5 spots across 3 pools). Correspondingly, patients N18 and N27 displayed longer RFS than N09 and N22 **(Figure 2a).** Taken together, the data from this cohort demonstrate that personalized neoantigen vaccination represents a promising approach for eliciting durable antitumor immune responses in HCC patients.

Immune microenvironment dynamics after neoantigen vaccination

To investigate changes in the tumor immune microenvironment induced by neoantigen vaccination, we conducted a comprehensive analysis of patient N22, from whom recurrent tumor tissue was obtained following vaccine treatment. Patient N22, a 46-year-old male, was initially diagnosed with HBV-associated HCC (MRI: 5.0 × 4.5 × 4.0 cm lesion in liver segment 5) at Mengchao Hepatobiliary Hospital of Fujian Medical University. He subsequently underwent curative resection followed by prophylactic TACE one month later. Pathology confirmed an invasive combined hepatocellular-cholangiocarcinoma (cHCC-CC) with stem cell features, accompanied by visible vascular tumor thrombi and circulating tumor cells, signifying elevated postoperative recurrence risk. Sequencing identified 44 candidate neoantigens from 85 nonsynonymous somatic mutations, of which 13 were successfully synthesized in time for inclusion in his customized neoantigen vaccine. Vaccination commenced three months post-surgery according to the scheduled prime-boost regimen **(Figure 3a).** One year after resection, MRI detected a 1.4 cm recurrent lesion at the original site, prompting a second hepatectomy. Immunohistochemistry demonstrated markedly higher infiltration of CD8+ T cells and elevated granzyme B expression in the recurrent tumor compared to the primary lesion **(Figure 3b).** The recurrent specimen underwent whole-exome and transcriptomic sequencing to evaluate vaccination effects on the immune landscape. Results revealed that mean variant allele frequencies of the 13 vaccinated neoantigen mutations decreased substantially—by 89% at the DNA level and 85% at the RNA level—in the recurrent tumor **(Figure 3c).** Similarly, frequencies of the remaining 72 somatic mutations declined (72% at DNA level; 67% at RNA level). Clonal evolution analysis between primary and recurrent tumors showed that 8 neoantigen mutations (5 others excluded due to copy number alterations) predominantly resided in clusters 2 and 3, which contracted significantly in the recurrence, while a novel clone (cluster 4) emerged, consistent with immune selection pressure driving tumor escape during neoantigen immunotherapy **(Figure 3d).** Additionally, 9 novel neoantigen mutations appeared in the recurrent tumor. These findings indicate that clones harboring vaccinated neoantigens were substantially reduced under immunotherapy pressure. Transcriptome-based immune infiltration profiling further revealed enriched effector populations in the recurrent tumor, including activated CD4+ T cells, activated CD8+ T cells, natural killer cells, and immature dendritic cells. Immunophenoscore comparison confirmed a shift toward a “hot” immune

microenvironment in the recurrence, characterized by improved MHC-mediated antigen presentation, greater effector T-cell abundance, and reduced T-cell immune checkpoint expression **(Figure 3e).** T-cell receptor (TCR) sequencing of both tumors identified two emergent clones (CASSESPLYEQYF and CASTTSGSYEQYF) exclusively in the recurrent tissue, indicative of vaccine-elicited neoantigen-specific T cells **(Figure 3f).** Collectively, these data demonstrate that neoantigen vaccination effectively stimulated antitumor T-cell responses capable of infiltrating the tumor bed.



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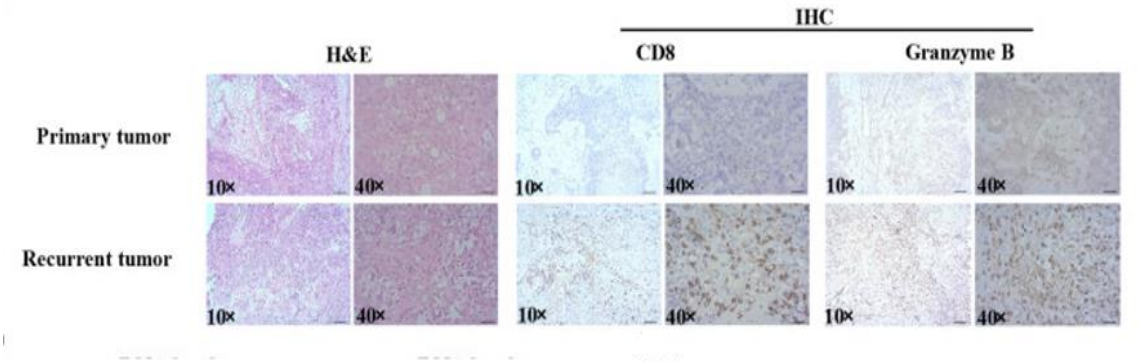

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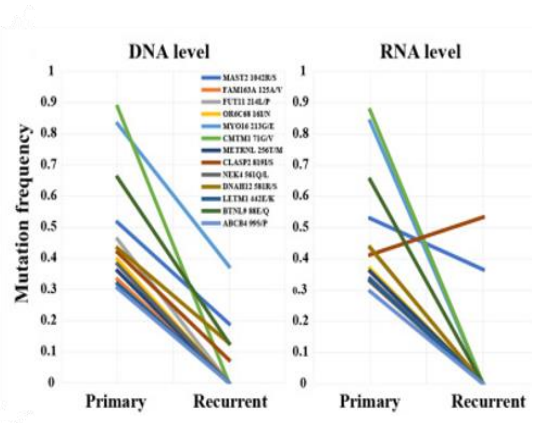

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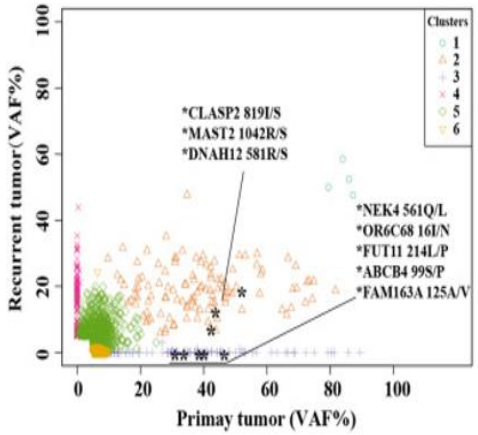

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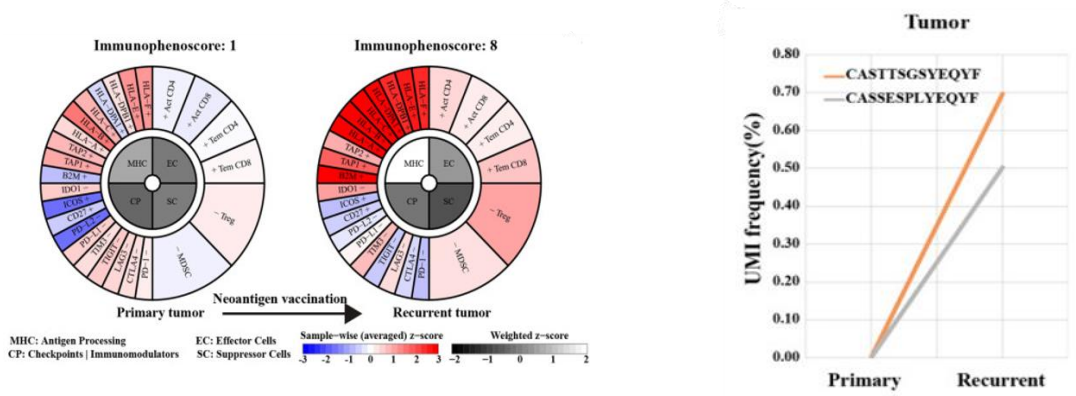
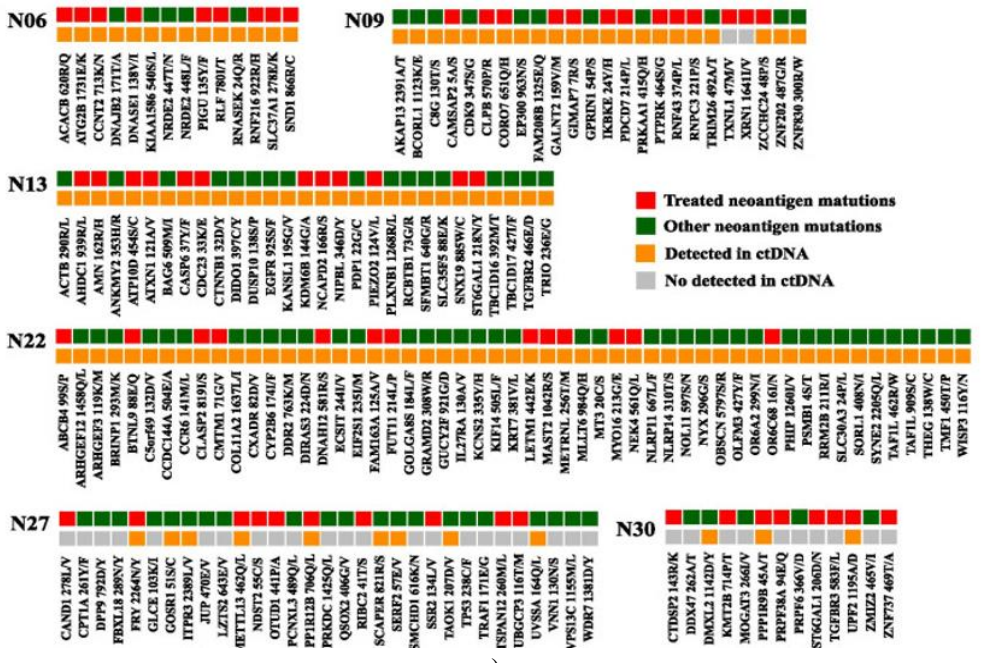


e) f)

Figure 3. Clinical response and immune microenvironment dynamics for HCC patients during neoantigen vaccination and follow up. a) Timeline of clinical events and corresponding imaging for patient N22 (day 1 defined as first vaccine dose). b) Hematoxylin-eosin staining alongside immunohistochemical detection of CD8 and granzyme B in primary and recurrent tumors. c) Variant allele frequencies of vaccinated neoantigen mutations at DNA and RNA levels in primary versus recurrent tumors. d) Clonal architecture evolution between primary and recurrent tumors (asterisks denote neoantigen mutations).e) Immunophenoscore profiles comparing primary and recurrent tumors. f) TCR repertoire dynamics between primary and recurrent tumors. VAF: variant allele frequency

The utility of ctDNA in assessing immune and clinical responses

Ongoing surveillance of both immune activation and clinical progress is crucial for gauging neoantigen vaccine performance and anticipating its benefits. Here, we followed treatment responses in 6 patients who finished the complete vaccination regimen (excluding N18 due to RNA editing-derived neoantigens) through serial detection of their unique nonsynonymous somatic mutations in ctDNA. Data in **Figure 4a** indicate that preoperative plasma samples had mean positivity rates of 74% for neoantigen loci (range 15.4~100%) and 78.9% for remaining somatic mutations (range 29~100%). In contrast, patients N27 and N30 displayed low detectability for most neoantigen and other mutation sites in ctDNA, rendering tumor burden monitoring unreliable over time. For the other 4 patients, variations in ctDNA levels of neoantigens and additional mutations closely paralleled tumor load estimates from CT/MRI scans across the vaccination and surveillance periods **(Figures 4b-4c)**.



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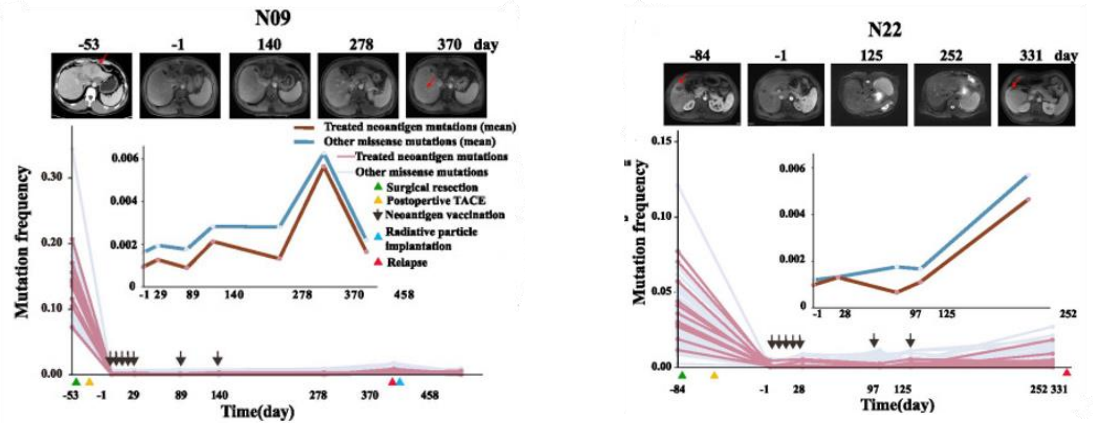

c) b)

Figure 4. Serial detection of individualized nonsynonymous somatic mutations in ctDNA throughout the treatment trajectory. a) Overview of neoantigen mutations and other somatic mutations identified in HCC specimens and corresponding preoperative plasma. Longitudinal quantification of vaccinated neoantigen mutations versus other somatic mutations in patient N09 (b) and N22 (c). Insets depict trends in average mutation allele frequencies for vaccinated neoantigen mutations and other somatic mutations over the course of vaccination and follow-up.

Furthermore, these 4 patients retained measurable postoperative ctDNA before vaccination onset, pointing to lingering minimal residual disease (MRD) despite radical resection and adjuvant TACE. Among the 2 patients with confirmed neoantigen immunity (N09 and N22), average total ctDNA levels (including vaccinated neoantigen mutations and others) climbed during the initial month covering the five prime doses, signaling advancing MRD **(Figures 4b-4c).** Intriguingly, later samples uncovered divergent ctDNA behaviors. From days 30 to 90, patient N09 exhibited drops in average frequencies of both vaccinated neoantigen ctDNA and non-targeted ctDNA; patient N22, however, showed a pronounced reduction solely in vaccinated neoantigen ctDNA, with non-targeted mutation frequencies continuing a mild upward trend. Subsequently, total ctDNA in patient N09 climbed between days 90 and 140, declined from day 140 to day 278 after the boosters, and rose once more leading to recurrence. Over the same interval, total ctDNA in patient N22 rose persistently despite boosters, reaching recurrence on day 331. Such divergence could stem from limited immune control during boosting or clonal selection enabling immune evasion amid neoantigen-targeted pressure. Notably, the progressive frequency shifts of neoantigen versus non-targeted mutations in patient N22's ctDNA aligned with observations in his recurrent tumor—specifically, elevated average frequencies of non-vaccinated mutations over vaccinated ones—indicating that vaccine-triggered immunity disproportionately cleared cells with targeted neoantigens, favoring expansion of alternative subclones. The remaining two patients (N13 and N06), who lacked evident neoantigen responses in PBMCs post-vaccination, displayed dissimilar ctDNA patterns. Overall, these observations validate that dynamic tracking of patient-specific neoantigen mutations in ctDNA offers a robust means for contemporaneous monitoring of immune activation and clinical status in HCC under neoantigen vaccination.

The elevated recurrence rate following curative resection remains the primary factor contributing to unfavorable outcomes in HCC patients. In China, prophylactic TACE represents the predominant approach to prevent recurrence, yet its use is surrounded by ongoing debate. Certain clinical investigations have reported that patients with portal vein tumor thrombosis (PVTT) undergoing prophylactic TACE post-resection experienced an approximate 2.4-month extension in recurrence-free survival compared to untreated counterparts, whereas other reports highlighted limited preventive benefits alongside notable toxicity [20–23]. Consequently, there is a pressing need to establish innovative, safe, and potent preventive therapies. Neoantigen-based vaccines, characterized by their targeted specificity, minimal adverse effects, and straightforward production, emerge as a promising option for recurrence prevention across various solid malignancies. Critically, surgical resection typically provides ample tumor and adjacent normal tissue, enabling thorough and precise identification of individual neoantigen profiles via next-generation sequencing. Additionally, postoperative patients generally exhibit slower disease advancement than those with advanced-stage illness, affording adequate time and resources for vaccine manufacturing. In the present trial, all 10 participants tolerated customized neoantigen vaccination well, with no significant adverse events observed after curative resection and prophylactic TACE. Their median

recurrence-free survival (RFS) post-surgery was 11.3 months. Notably, the 5 patients who mounted robust neoantigen-specific responses following complete vaccination achieved extended RFS relative to non-responders, individuals receiving only prime doses, and propensity score-matched controls. Given the modest cohort size, larger studies are required for confirmation. Thus, neoantigen vaccination holds promise as a viable preventive modality in HCC.

Furthermore, continuous assessment of clinical response to neoantigen vaccination is indispensable for guiding physician decisions. Conventional serum markers and imaging modalities suffer from inadequate sensitivity and specificity, rendering them unsuitable for tracking responses in patients with undetectable tumor burden during immunotherapy. In this trial, we pioneered real-time evaluation of vaccine efficacy by serially detecting patient-specific neoantigen mutations alongside other somatic alterations in plasma ctDNA. We identified two distinct ctDNA kinetic profiles: one where neoantigen mutation dynamics mirrored those of non-targeted mutations throughout vaccination, suggestive of low intratumoral heterogeneity—implying widespread presence of targeted neoantigens across tumor cells and effective immune-mediated clearance by vaccine-activated T cells, leading to parallel declines in both mutation types. The alternative profile showed discordant patterns between neoantigen and other mutations, indicative of substantial heterogeneity, with targeted neoantigens confined to a subset of clones. These observations furnish reliable indicators for gauging treatment response and durability of neoantigen immunotherapy, facilitating timely adjustments to management plans.

Additionally, ctDNA data revealed that the antitumor immunity elicited by neoantigen vaccination remains transient, insufficient to fully suppress minimal residual disease (MRD) advancement. Potential explanations include constraints on the number of neoantigens incorporated—although many candidates were identified in some cases, only up to 20 could be synthesized due to time and economic factors—and the inherent heterogeneity of HCC, allowing outgrowth of subclones lacking vaccine-targeted neoantigens that evade recognition by induced T cells. Hence, enhancing preventive potency and response longevity necessitates refinement of vaccination protocols and/or integration with complementary therapies, such as immune checkpoint blockade or tyrosine kinase inhibitors [24]. Emerging evidence suggests that combining neoantigen vaccines with PD-1 inhibitors augments antitumor activity in malignancies like melanoma and non-small cell lung cancer [25], though validation in HCC is awaited.

Conclusion

In conclusion, neoantigen vaccination proves safe, practical, and efficacious for preventing HCC recurrence after curative surgery. Serial monitoring of individualized neoantigen mutations in ctDNA delivers sensitive and specific insights into treatment response, thereby supporting personalized therapeutic strategies. Nonetheless, limitations persist: the absence of custom neoantigen tetramers for detailed T-cell characterization due to resource constraints, and the small cohort size necessitating corroboration in larger prospective trials.

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