

# In Situ Vaccination with Poly-ICLC Combined with Systemic Nivolumab for the Treatment of Unresectable Hepatocellular Carcinoma

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**Purpose:** Unresectable hepatocellular carcinoma (HCC) presents significant therapeutic challenges. While immune checkpoint inhibitors (ICIs) are part of the current standard of care, combining poly-ICLC as an in situ vaccination with an ICI may enhance treatment efficacy. The study investigated the safety and therapeutic effects of combining poly-ICLC with nivolumab, an ICI, in patients with unresectable HCC.

**Patients and Methods:** Patients with unresectable HCC were enrolled to receive intratumoral and intramuscular poly-ICLC injections along in combination with nivolumab infusions. The primary endpoint was safety, and secondary endpoints included objective response as measured by mRECIST and changes in serum alpha-fetoprotein (AFP) levels. Gene expression profiling, pathway analysis, and immune cell type deconvolution were conducted using NanoString GeoMx Digital Spatial Profiling.

**Results:** Four patients were enrolled. The combination therapy was safe and well-tolerated. Among them, one patient achieved a complete response (CR), and another achieved a partial response (PR). Both responders showed significant declines in serum AFP levels. Notably, the patient with CR showed eradication of cancerous component of the portal vein thrombus, and an abscopal effect was observed in the patient with PR. Gene analysis indicated that interferon-gamma signaling was the most enriched pathway in tumors of the responders.

**Conclusion:** This combination therapy was safe and effective, with two out of four patients demonstrating objective responses. These preliminary findings warrant further investigation into larger clinical cohorts.

**Keywords:** Hepatocellular carcinoma, Poly-ICLC, In situ vaccination, immune checkpoint inhibitors, Portal vein thrombus, Digital spatial profiling

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide.<sup>1</sup> Early-stage HCC can be effectively treated with surgical resection, radiofrequency ablation, or liver transplantation.<sup>2</sup> However, despite the implementation of screening programs for high-risk populations, a significant proportion of patients are still diagnosed at an advanced stage.<sup>3</sup> For these patients, systemic therapy is essential. The first major breakthrough in systemic therapy came in 2007 with the introduction of molecular targeted therapies, led by sorafenib. In 2011, the emergence of immune checkpoint inhibitors (ICIs) marked another important advance in HCC treatment. Nivolumab, an anti-PD1 antibody, was

approved in 2017 as a second-line monotherapy for advanced HCC.<sup>4</sup> Subsequent studies demonstrated that ICI-based combination therapies often outperform ICI monotherapy.<sup>5</sup> Currently, the combination of atezolizumab (anti-PD-L1) and bevacizumab (anti-VEGF) is considered the most effective first-line treatment for unresectable HCC, showing an objective response rate (ORR) of 30% and a median overall survival of 19.2 months—surpassing the results achieved with sorafenib.<sup>6</sup> Nevertheless, there remains a need to further improve therapeutic efficacy, and numerous trials are ongoing to explore novel ICI-based combinations.<sup>4</sup>

Therapeutic cancer vaccines have emerged as promising candidates for combination with ICIs, given their complementary mechanisms of action in the cancer–immunity cycle. Cancer vaccines function by increasing the anti-tumor T cell repertoire: they promote antigen release from tumor cells and facilitate antigen presentation to the immune system. In contrast, ICIs restore T cell activity by blocking immune checkpoints that suppress T cell function.<sup>7</sup> This mechanistic complementarity suggests a potential for synergistic therapeutic effects when the two are combined.<sup>8</sup> Cancer vaccines can target a range of antigens, including tumor-specific antigens—such as viral antigens and neoantigens arising from non-synonymous somatic mutations—and tumor-associated antigens, which include tissue- or development-specific antigens.<sup>9</sup> Despite decades of research, vaccines targeting tumor-associated antigens have yielded limited clinical success.<sup>10</sup> More recently, interest has shifted toward personalized neoantigen vaccines.<sup>9</sup> Although T cell responses have been elicited in clinical trials of neoantigen-based vaccines, these have not always translated into meaningful clinical benefit.<sup>11,12</sup> However, promising developments have emerged. A recent clinical trial using an mRNA-based individualized neoantigen vaccine in patients with resected melanoma demonstrated that combining the vaccine with pembrolizumab (anti-PD1) significantly prolonged recurrence-free survival and reduced recurrence rates compared to pembrolizumab monotherapy.<sup>13</sup> Another single-arm trial in HCC evaluated DNA plasmid-encoded neoantigens combined with plasmid-encoded interleukin-12 and pembrolizumab, reporting an ORR of 30.6%, with 8.3% of patients achieving a complete response (CR).<sup>14</sup> These findings support further clinical investigation into vaccine-ICI combinations.

Nevertheless, neoantigen vaccine strategies face challenges, particularly due to tumor genetic heterogeneity, which is a key factor in resistance to cancer therapies.<sup>15,16</sup> Neoantigens derived from one tumor region may not be expressed in others, limiting the effectiveness of such vaccines. Additionally, personalized neoantigen vaccine development is time-consuming and costly, requiring high-throughput sequencing, complex bioinformatics, antigenicity prediction, and customized peptide or mRNA synthesis. An alternative and potentially more practical strategy is *in situ* vaccination (ISV). ISV leverages therapy-induced immunogenic cell death (ICD) to trigger the release of tumor antigens, followed by activation of local antigen-presenting cells (APCs), which then stimulate a tumor-specific immune response.<sup>9,17</sup> ISV-generated vaccines are both personalized and off-the-shelf, providing logistical advantages.<sup>18</sup> Moreover, ISV enables repeated administration across multiple tumor sites or distinct tumors, potentially overcoming challenges posed by tumor heterogeneity.

One promising candidate for ISV is polyinosinic–polycytidylic acid–poly-L-lysine carboxymethylcellulose (poly-ICLC). This synthetic, virus-mimicking double-stranded RNA activates both Toll-like receptor (TLR) 3 and melanoma differentiation-associated gene 5, triggering robust immune responses.<sup>19</sup> In our previous studies, *in vitro* incubation of poly-ICLC induced ICD in HCC cells, and intratumoral (IT) injection of poly-ICLC activated endogenous dendritic cells (DCs), the most potent APCs. Sequential IT followed by intramuscular (IM) poly-ICLC administration significantly suppressed HCC growth and enhanced infiltration of CD8<sup>+</sup> T cells in two syngeneic murine HCC models.<sup>20</sup> Furthermore, we demonstrated that combining either IT or IM administration—particularly IT—of poly-ICLC with systemic anti-PD1 therapy synergistically inhibited tumor growth in a mouse HCC model.<sup>21</sup>

A clinical trial has investigated the use of IT and IM poly-ICLC injections in combination with other therapeutic modalities, such as irradiation and local regional treatment, to treat patients with unresectable HCC in the pre-ICI era. Some patients demonstrated tumor responses and prolonged survival.<sup>22</sup> Building on our previous animal study demonstrating the synergistic inhibitory effect of poly-ICLC combined with anti-PD1 on HCC growth,<sup>21</sup> we conducted this clinical trial to evaluate the combination of poly-ICLC and nivolumab in patients with unresectable HCC.

## Materials and Methods

### Study Design

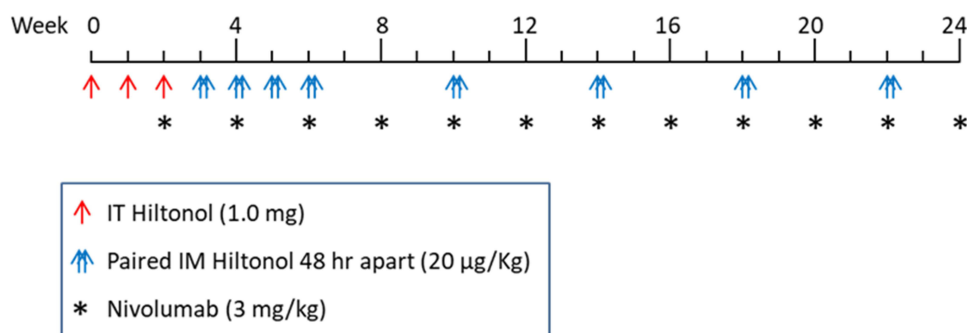
This open-label, single-arm clinical trial was conducted at National Taiwan University Hospital, Taipei, Taiwan. The study aimed to evaluate both safety and tumor response, as assessed using the modified Response Evaluation Criteria in Solid Tumors (mRECIST).<sup>23</sup> The trial investigated the combination of IT and IM poly-ICLC injections (Oncovir, Inc., Washington, DC, USA) with nivolumab (Bristol-Myers Squibb, Princeton, NJ) in patients with unresectable HCC.

In patients with multinodular HCC, a single tumor larger than 2 cm and accessible for ultrasound-guided IT injection was designated as the target lesion. A needle liver biopsy of the target tumor was performed within 28 days prior to initiating therapy. Patients received weekly IT injections of poly-ICLC (1.0 mg) for three weeks, followed by biweekly IM booster injections (20 µg/kg) for four weeks. This was succeeded by four additional cycles, each consisting of a three-week rest period and two biweekly IM injections. The IT and IM dosing regimens were based on previous studies.<sup>22</sup> Nivolumab was administered intravenously at a dose of 3 mg/kg over 90 minutes every two weeks, beginning in week 2, for a total treatment duration of six months (see Figure 1). Following treatment completion, patients were monitored for up to two years or until withdrawal, death, or other study exit criteria were met. CT scans of the brain, chest, abdomen, and pelvis were conducted at baseline, as well as at 12 and 24 weeks post-treatment initiation. A radiologist (P.C.L.) measured the CT attenuation values (in Hounsfield units, HU) of portal vein thrombosis (PVT) regions, calculating the contrast enhancement by comparing pre- and post-contrast images. All research procedures adhered to the Declaration of Helsinki. The study protocol was approved by the Research Ethics Committee of National Taiwan University Hospital (approval number: 202104104MIND) and registered at ClinicalTrials.gov (identifier: NCT05281926).

### Patient Eligibility

Participants were eligible for enrollment if they met the following inclusion criteria: (1) histologically confirmed diagnosis of HCC, (2) age 20 years or older, (3) unresectable disease assessed by the surgeon Y.M.W., (4) radiologically measurable disease with a target tumor at least 2 cm in the longest dimension, (5) ECOG performance status of  $\leq 2$ , (6) Child-Pugh classification A, (7) for patients with chronic hepatitis B (HBV) infection, long-term antiviral agents with a high barrier to resistance, (8) for patients with chronic hepatitis C (HCV) infection, achievement of sustained viral response with any direct-acting agent, (9) acceptable hematologic, renal, and liver function within 28 days before trial entry (absolute neutrophil count  $\geq 1500/\text{mm}^3$ , platelets  $\geq 80,000/\text{mm}^3$ , hemoglobin  $> 10.0 \text{ g/dL}$ , creatinine  $\leq 2.0 \text{ mg/dL}$ , total bilirubin  $\leq 1.5 \text{ mg/dL}$  unless due to Gilbert's syndrome, AST and ALT  $\leq 2$  times the upper limits, INR  $< 1.5$ , and (10) ability to provide informed consent.

Patients were excluded if they met any of the following conditions: (1) concurrent use of other investigational agents, biological agents, or anti-cancer medications, (2) uncontrolled intercurrent illnesses, including ongoing or active infection requiring antibiotics, symptomatic congestive heart failure, unstable angina pectoris, or psychiatric/social situations that would limit compliance with study requirements, (3) pregnancy or nursing, (4) diagnosis of primary immunodeficiency diagnosis or use of systemic steroids or other immunosuppressive therapy within 7 days prior to the



**Figure 1** Schematic representations of treatment protocol.

first dose of trial treatment, (5) active autoimmune disease requiring systemic treatment in the past year, (6) HIV-positive patients with detectable viral load, those not on a stable HAART regimen, or with  $<200$  CD4<sup>+</sup> T cells/microliter in peripheral blood, (7) history of allogeneic hematopoietic cell transplantation or solid organ transplantation, and (8) documented allergy or hypersensitivity to any protein therapeutics.

## Assessments of Tumor Response and Toxicity

Tumor response was evaluated via CT imaging using mRECIST criteria at screening, week 12, and at the end of treatment. Serum alpha-fetoprotein (AFP) levels were monitored every two months. Adverse events (AEs) were graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 5.0.

Dose-limiting toxicities (DLTs) related to poly-ICLC, specifically those classified as Grade 3 or 4, were closely monitored. If a DLT occurred, treatment was paused until the toxicity resolved to Grade 1 or baseline. Upon resolution, a reduced poly-ICLC dose (50%) was administered. If the DLT did not resolve, the patient was withdrawn from the study.

## Slide Preparation and Staining

Formalin-fixed, paraffin-embedded biopsy tissues were serially sectioned at 5  $\mu$ m thickness for 5 sections and mounted onto slides. The slides were then baked in a histology oven for 1 hour. After deparaffinization and rehydration in ethanol gradient, the first slide underwent hematoxylin and eosin (H&E) staining to serve as the reference slide. The diagnosis and assessment of HCC differentiation according to the Edmondson-Steiner grading system were conducted by pathologist C.T.S. The other slides underwent double immunofluorescence staining using primary fluorophore-conjugated antibodies targeting cytokeratin (CK) 8 (Novus Biologicals, NBP2-34655AF488, k8.8+DC10,1:100 dilution to yield green color), CD45 (Novus Biologicals, NBP2-34528AF594,2B11+PD7/26,1:100 dilution to yield red color) plus Syto 83 nuclei dye. One of the slides with best quality was selected for digital spatial profiling (DSP).

## Selection and Segmentation of Regions of Interest for DSP

In the GeoMx DSP instrument, the selected slides of each sample were imaged at 20X magnification and 3 regions of interest (ROIs) of CK8<sup>+</sup> tumor nests and 3 ROIs of CD45<sup>+</sup> tumor microenvironment (TME) in the vicinity of the tumor nests were drawn with random shapes. In the special case of infiltrative-type HCC (Patient 1), the tumor nests (labeled as “T”) were interwoven with normal parenchyma (labeled as “NP”). Both regions were CK8<sup>+</sup> and thus difficult to distinguish on the immunofluorescence-stained slide (Figure 2d). To avoid selecting CK8<sup>+</sup> normal parenchyma, the CK8<sup>+</sup> tumor nests (T1 and T2) were identified with the guidance of the H&E-stained reference slide (Figure 2a). Enlarged images of the T1 and T2 tumor nests in the H&E-stained slide are shown in Figures 2b and c, respectively. In these images, the tumor regions are clearly distinguishable from the normal parenchyma based on nuclear atypia, including enlarged nuclei, hyperchromasia, a higher nuclear-to-cytoplasmic ratio, and pleomorphism. The GeoMa software was then used to segment the tumor ROIs to red color CK8<sup>+</sup> and neighboring green color CD45<sup>+</sup> regions (Figure 2e and f). The ROIs were then subjected to UV illumination at 385 nm. The programmable digital micromirror device embedded in the Geo Mx DSP machine allows for selective illumination. UV cleavable oligonucleotides were released and collected for subsequent library preparation.

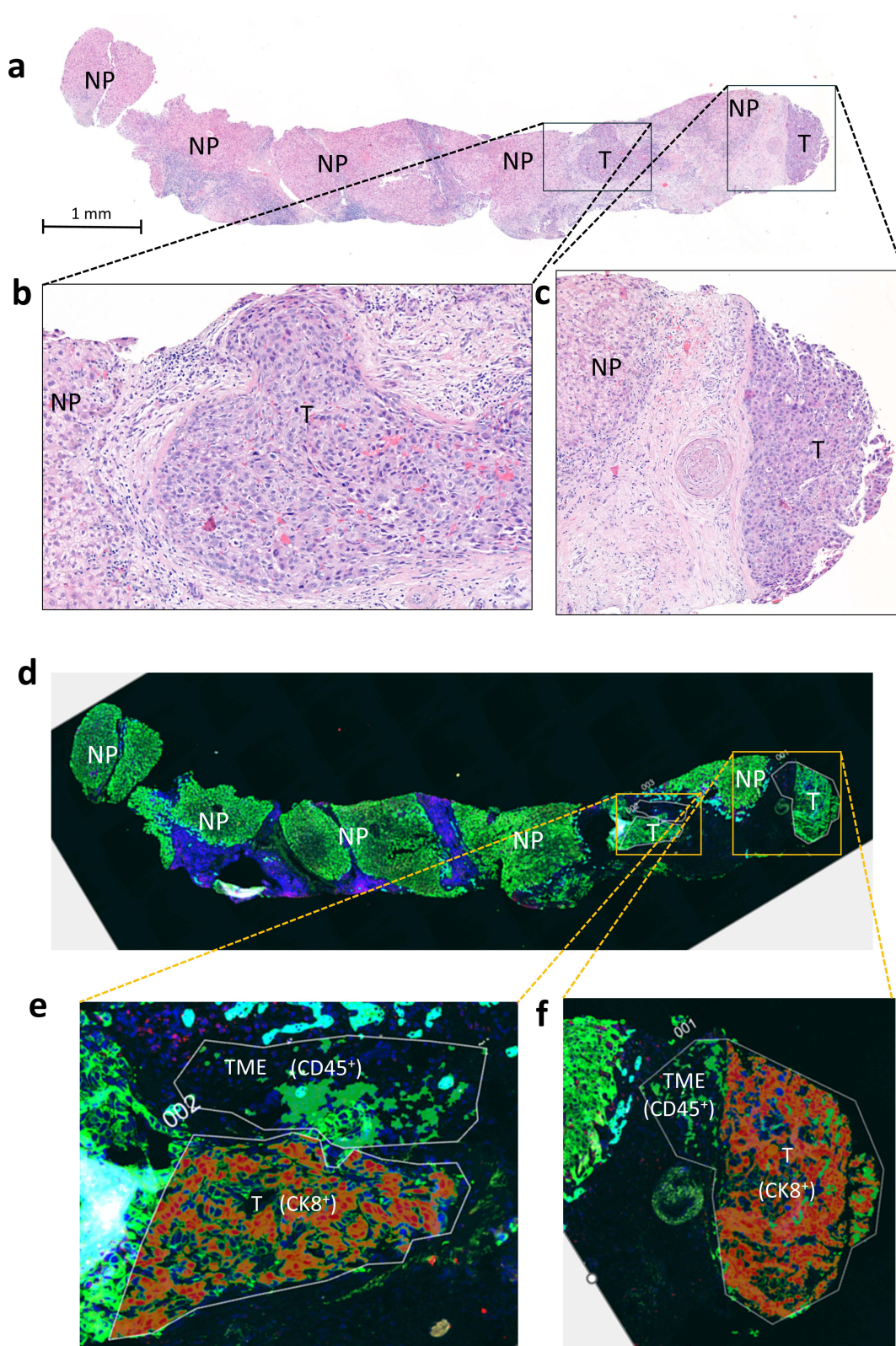
## Library Preparation and Sequencing

Sequencing libraries were prepared via polymerase chain reaction using photo-released indexing oligonucleotides and area of illumination (AOI)-specific illumination adapter sequences. The resulting libraries were sequenced on an Illumina NovaSeq 6000 platform.

## Analysis Description

Initial quality control checks were performed on the raw count data. Genes with expression levels comparable to the geometric mean of negative control probes in more than 95% of AOIs were filtered out. Following this step, 12,322 genes were retained from the original 18,676. The remaining data were normalized using the third quartile (Q3) normalization method. Differential





**Figure 2** Selection and segmentation of ROIs in CK8<sup>+</sup> and CD45<sup>+</sup> regions using Patient I as an example. (a) H&E staining of a serially sectioned needle liver biopsy tissue, delineating tumor and normal parenchyma regions. (b and c) Magnified views of the areas marked in a. (d) Selection of CK8<sup>+</sup> cells (green) in the HCC immunofluorescence image, referencing the serial sections in a to avoid including NP. Neighboring CD45<sup>+</sup> cells (red) were also selected. (e and f) Magnified images of the selected CK8<sup>+</sup> and CD45<sup>+</sup> ROIs with CK8<sup>+</sup> cells represented in red and CD45<sup>+</sup> cells in blue. Scale bar= 1 mm; T= tumor; NP= normal parenchyma.

expression analysis was conducted using an unpaired *t*-test with p-values adjusted by the Benjamini-Hochberg false discovery rate (FDR) method. Normalized gene expression data were compared based on AOI annotations. Pathway enrichment analysis was performed using the fGSEA package integrated into the NanoString DSP Analysis Suite. Pathways with an adjusted p-value  $\leq 0.05$  were considered significant and ranked by their Normalized Enrichment Score (NES). Cell type deconvolution was carried out using the Spatial Decon package, leveraging the Immune Tumor Safe TME signature profile.

## Results

### Patient Enrollment

Enrollment of eligible patients began in September 2022. At that time, Taiwan’s National Health Insurance (NHI) did not provide coverage of ICIs for patients with advanced HCC, making the provision of nivolumab in this trial a significant incentive for enrollment. However, in August 2023, Taiwan’s NHI began reimbursing the combination of atezolizumab and bevacizumab for these patients. Consequently, the trial was prematurely terminated due to the loss of this enrollment incentive and ethical concerns, as there remained no rationale to suggest the investigational regimen offered greater benefit than the now-available standard treatment.

### Patient Characteristics

Only four patients were enrolled before the trial was terminated, with a mean age of 75.8 years (range 70–80), including three men and one woman. Two patients had HBV-related HCC and two had HCV-related HCC. Three patients had liver cirrhosis, all classified as Child–Pugh A. Pre-treatment albumin levels were normal in all patients, indicating preserved liver reserve. Based on the Barcelona Clinic Liver Cancer (BCLC) staging system, two patients were stage B and two were stage C. Three had a history of HCC treated with curative resection but had not received therapy for their recurrent disease prior to enrollment. The current tumors were infiltrative in one patient (Patient 1) and multinodular in the other three. Tumor differentiation, graded by the Edmondson-Steiner system, with three patients classified as grade 3 and one as grade 4. In the multinodular cases, the number of nodules ranged from 3 to over 10, with maximum diameters between 2 and 4.7 cm. Although CT scans revealed no extrahepatic spread, portal vein thrombosis (PVT) was present in two patients (Patient 1 and 4). Notably, these two patients with PVT also had serum AFP levels exceeding 400 ng/mL, suggesting that elevated AFP may correlate with more aggressive tumor characteristics in advanced HCC (Table 1).

**Table 1** Patient Characteristics, Tumor Features, and Outcomes

Patient Number	1	2	3	4
Age (yr)	78	75	80	70
Sex	M	M	F	M
Etiology	HCV	HBV	HCV	HBV
BCLC stage	C	B	B	C
Prior HCC	Yes	Yes	Yes	No
Prior therapy	SR	SR	SR	No
Cirrhosis*	Yes	No	Yes	Yes
Tumor feature	IF	MN	MN	MN
E-S grade	3	3	3	4
Tumor number	1	6	>10	3
Maximum diameter (cm)	2.4	3.1	2	4.7
Extrahepatic spread	No	No	No	No
PVT	Yes	No	No	Yes
Trial duration (month)	6	6	3	6
Alb (g/dL)	4.4	4.4	4.1	3.9

(Continued)

**Table 1** (Continued).

Patient Number	1	2	3	4
Initial AFP (ng/mL)	2490	4.76	113	419
Post-trial AFP (ng/mL)	2.11	5.35	1593	22
Treatment response	CR	PD	PD	PR

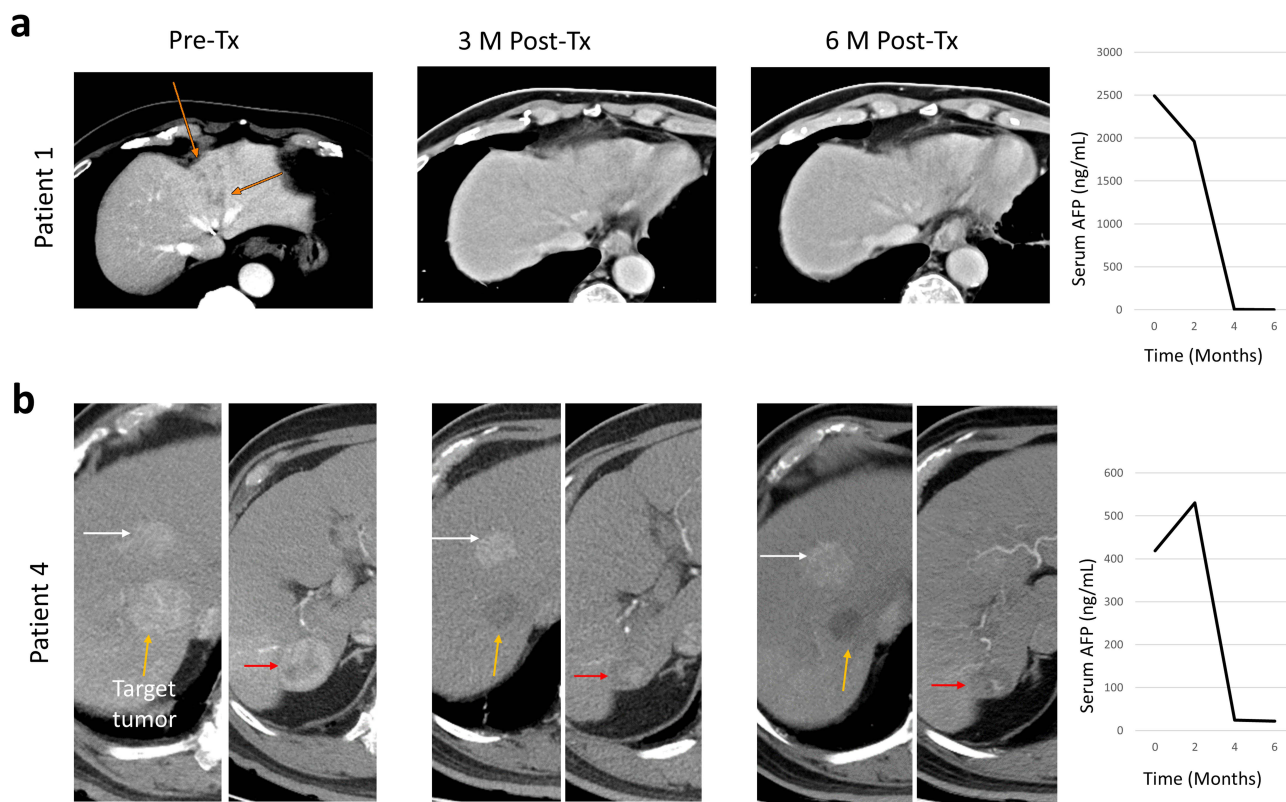
**Note:** \*All the cirrhosis was in Child-Pugh classification A.

**Abbreviations:** M, male; F, female; HCV, hepatitis C virus; HBV, hepatitis B virus; SR, surgical resection; IF, infiltrative; MN, multinodular; E-S, Edmondson-Steiner; PVT, portal vein thrombosis; Alb, albumin; AFP, alpha-fetoprotein; CR, complete response; PR, partial response; PD, progressive disease.

## Patient Outcomes Revealed Varied Responses

Three patients (Patients 1, 2, and 4) completed the full treatment course, while one patient (Patient 3) withdrew early due to rapid disease progression. At trial entry, Patient 3 had more than ten lesions and showed aggressive disease progression, prompting discontinuation of the study treatment after three months. She subsequently received one session of transarterial chemoembolization (TACE) and lenvatinib, but her condition continued to worsen, culminating in the development of ascites, hepatic encephalopathy, liver failure, and ultimately, death. Patient 2, who initially presented with six tumors, did not exhibit a treatment response (Table 1).

Of the three patients who completed the trial, two (Patients 1 and 4) demonstrated favorable responses. Both had PVT. Patient 1 had infiltrative HCC with bilateral PVT; following treatment, the infiltrative lesion resolved completely (Figure 3a). Patient 4 initially presented with three tumors and right-sided PVT. By the end of the study, the target lesion



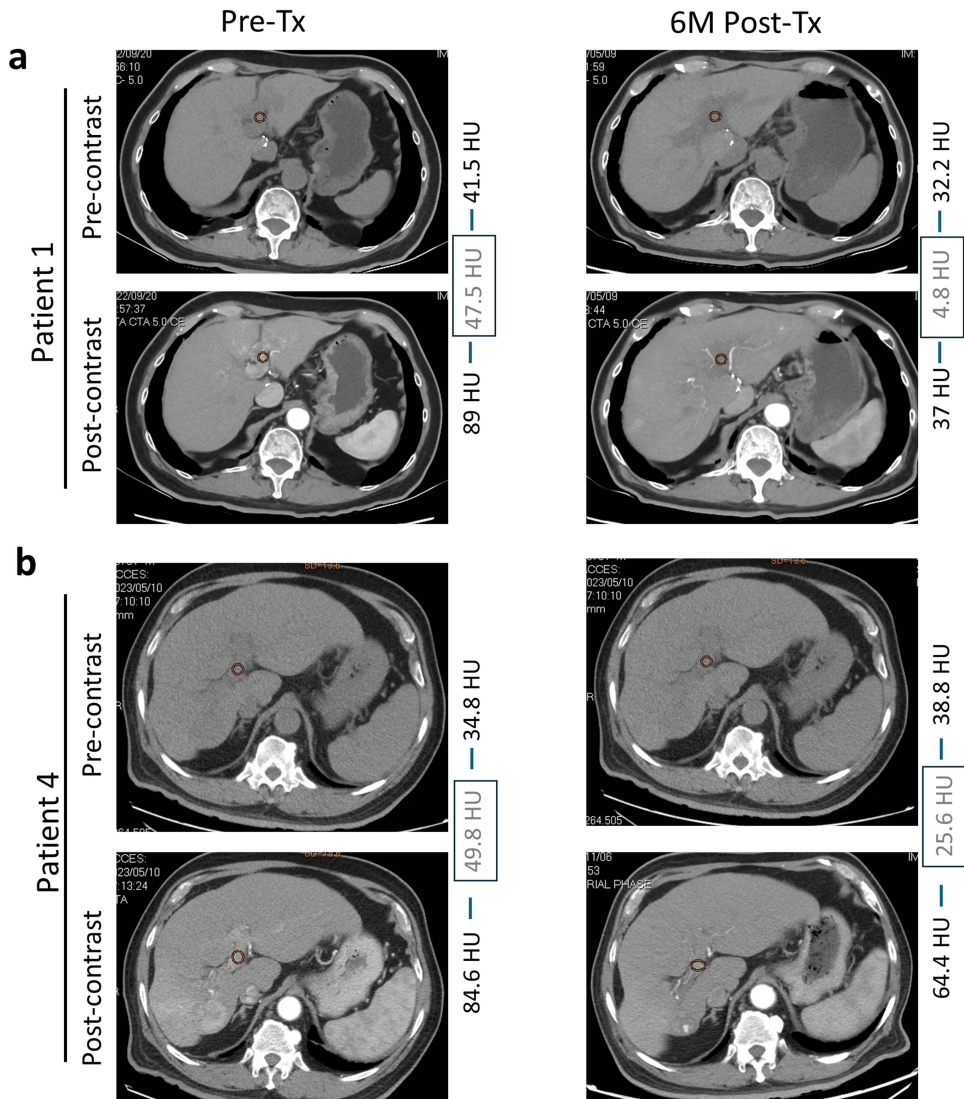
**Figure 3** Tumor response after treatment in Patient 1 and Patient 4. (a) In Patient 1, pre-treatment CT in the venous phase shows scattered ill-defined hypoattenuating lesions (arrows) in the medial segment, which are absent in post-treatment CTs. (b) In Patient 4, serial CTs in the arterial phase demonstrate gradual regression of the target tumor (yellow arrows), which became nearly unenhanced after 6 months of treatment. Another nodule (red arrows) also regressed and exhibited decreasing enhancement, while a third nodule (white arrows) showed no signs of regression. Serum AFP levels in both patients declined significantly following treatment. Pre-Tx= Pre-treatment; 3M Post-Tx= 3 months post-treatment; 6M- post-Tx= 6 months post-treatment.



showed significant shrinkage and a marked reduction in contrast enhancement on CT imaging (yellow arrows, [Figure 3b](#)). Notably, one of the non-injected tumors also exhibited a decrease in both size and enhancement (red arrows, [Figure 3b](#)), while another increased in size and retained its enhancement (white arrows, [Figure 3b](#)). Although PVT persisted in both responders, their serum AFP levels declined markedly ([Table 1](#); [Figure 3a](#) and [b](#)).

In the regions of PVT, the pre-treatment increase in CT attenuation from pre-contrast to post-contrast scans was 47.5 hU for Patient 1 and 49.8 hU for Patient 4. By the end of treatment, these values had dropped to 4.8 hU and 25.6 hU, respectively ([Figure 4](#)). Based on established criteria defining portal vein tumor thrombosis (PVTT) as a  $\geq 20$  hU increase,<sup>24,25</sup> Patient 1's PVT was classified as PVTT at baseline but was reclassified as bland post-treatment. In contrast, Patient 4's PVT met the criteria for PVTT both before and after treatment; however, the reduced enhancement (from 49.8 to 25.6 hU) suggests a response of the PVTT to therapy.

In summary, Patient 1 achieved a CR, with the disappearance of infiltrative HCC and resolution of PVTT. Patient 4 achieved a partial response (PR), with more than a 30% reduction in total intrahepatic tumor diameter and decreased enhancement of residual PVTT, which remained neoplastic ([Table 1](#)).



**Figure 4** CT numbers in the PVT areas expressed as HU for Patient 1 (**a**) and Patient 4 (**b**). Pre-contrast and post-contrast CT numbers were measured, with the increase from pre- to post-contrast noted in the boxes. Small circles indicate the specific areas in the PVT where CT numbers were measured. Pre-Tx= Pre-treatment; 6M- post-Tx= 6 months post-treatment.



**Table 2** Adverse Events

Patient Number	1	2	3	4
Fever	Yes/2*	Yes/1	No	Yes/1
Rash	Yes/1	Yes/1	No	Yes/1
Anorexia	Yes/2	No	Yes/1	Yes/1
Abdominal pain	No	Yes/1	No	No
Arthralgia	Yes/1	No	No	No
Constipation	No	No	No	Yes/1
Elevation of transaminases	No	No	No	Yes/1

**Note:** \*The number after the slash indicates the AE grade.

## No Severe Toxicities Were Reported After Treatment

AEs were generally mild. Patients 1, 2 and 4 experienced grade 1–2 fevers after IT injections of poly-ICLC, all of which resolved uneventfully with antipyretic treatment. No fever was reported after IM injections. Other treatment-related AEs—including skin rash, anorexia, abdominal pain, arthralgia, constipation, and elevated serum transaminases—were all grade 2 or lower in severity (Table 2).

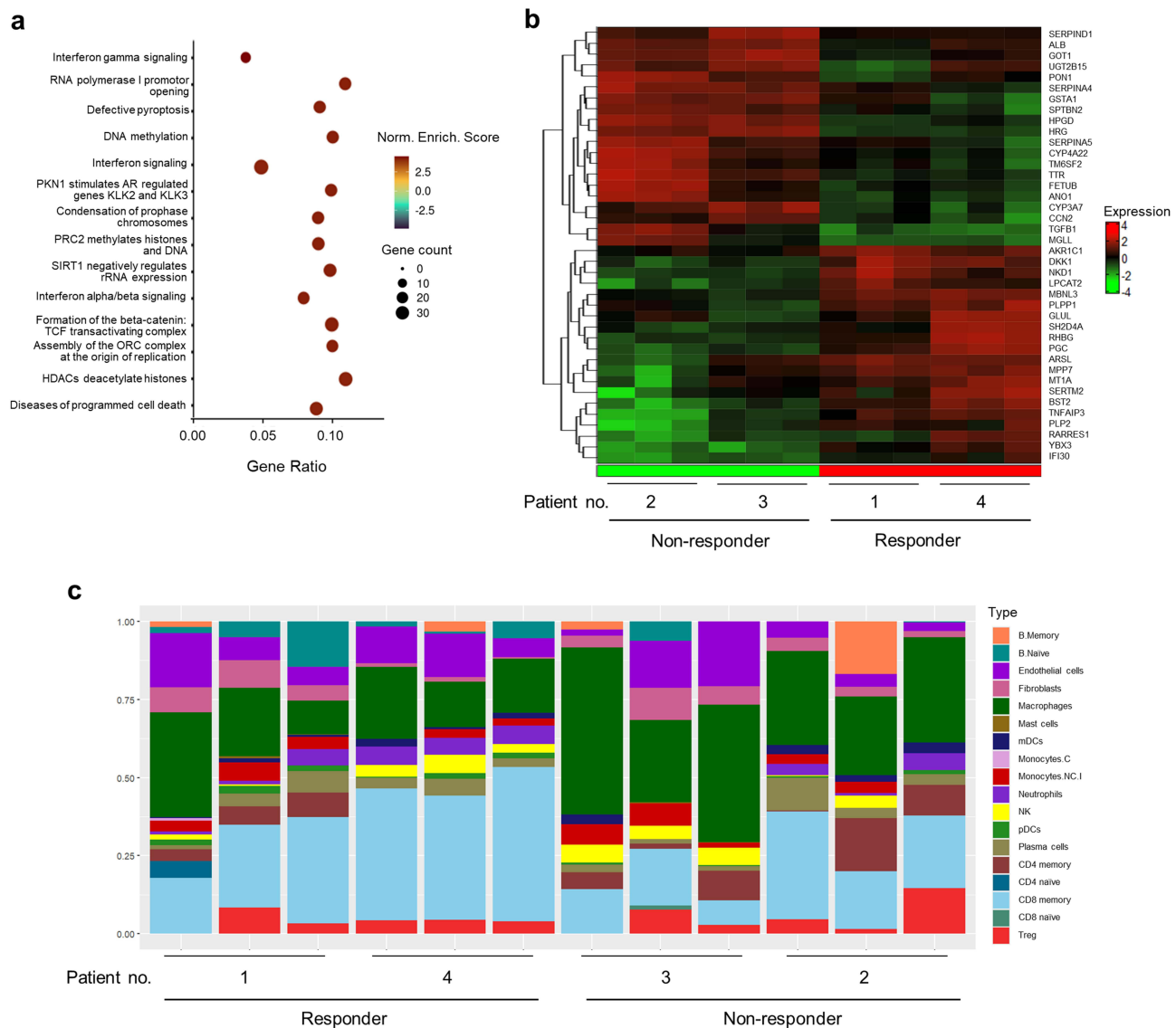
## Responders Exhibited Enriched Interferon Signaling and CD8 Memory T Cell–Rich Tumor Microenvironment

Pathway enrichment analysis of tumor cells revealed significant upregulation of both interferon-gamma (IFN- $\gamma$ ) and interferon-alpha/beta signaling in treatment responders (Figure 5a). Supporting this, a heatmap analysis showed elevated expression of interferon gamma-inducible protein 30 (IFI30) in the responder group (Figure 5b). Immune cell deconvolution further highlighted a predominance of CD8 memory T cells in the tumor microenvironment of responders, whereas non-responders showed greater infiltration by CD4 memory T cells and macrophages (Figure 5c).

## Discussion

Despite the number of enrolled patients was low, this study demonstrated that the combination therapy of poly-ICLC and anti-PD1 was well tolerated and safe, with only low-grade AEs. Reported AEs included fever and skin rash (Patients 1, 2 and 4), anorexia (Patients 1, 3 and 4), abdominal pain (Patient 2), arthralgia (Patient 1), and constipation and elevated serum transaminases (Patient 4). All AEs were grade 2 or lower in severity (Table 2). Two patients showed promising responses to the therapy—one CR (Patient 1) and one PR (Patient 4) (Table 1)—highlighting the potential efficacy of this combination approach in treating HCC. All participants were over 70 years of age (Table 1), an age group commonly associated with immunosenescence, which can reduce the efficacy and safety of immunotherapies.<sup>26</sup> However, such age-related limitations were not observed in this study. Whether younger patients might derive even greater benefit from this regimen remains an open question.

Lurje et al previously advocated for the use of ISV as a therapeutic strategy against HCC.<sup>17</sup> ISV promotes systemic anti-tumor immunity by inducing ICD in tumor cells, which recruits DCs to the tumor site, facilitates antigen uptake, and activates DCs to present tumor neoantigens, thereby priming tumor-specific CD8<sup>+</sup> T cells in the draining lymph nodes (dLNs).<sup>27</sup> Modalities such as radiotherapy, chemotherapy, TACE, and targeted therapies have been explored for ISV,<sup>16</sup> as they can induce ICD and remodel the TME, but their ability to recruit and activate DCs is often limited.<sup>17</sup> Immunological adjuvants, particularly TLR ligands like poly(I:C), LPS, CpG, R848, and Pam3CSK4, have demonstrated greater potential in this regard.<sup>28</sup> Our previous murine studies confirmed that poly-ICLC induced ICD on HCC cell lines and activated DCs in both the tumors<sup>20</sup> and dLNs.<sup>21</sup> Sequential IT followed by IM administration of poly-ICLC inhibited tumor growth and induced an abscopal effect.<sup>20</sup> Additionally, combining poly-ICLC (via either IT or IM injection) with anti-PD1 therapy led to greater anti-tumor efficacy compared to anti-PD1 monotherapy, supporting the role of poly-ICLC as an immunostimulatory enhancer of checkpoint blockade.<sup>21</sup> Although poly-ICLC had previously been evaluated in a Phase 1 clinical trial as an ISV agent before the widespread use of ICIs,<sup>22</sup> its combination with anti-PD1 has not yet



**Figure 5** Gene expression analyses and immune cell deconvolution by DSP. (a) Top 14 enriched pathways in responders including type II interferon pathway (Interferon gamma signaling) and type I interferon pathway (Interferon alpha/beta signaling). (b) Top 20 differentially expressed genes in responders and top 20 in non-responder. (c) Immune cell deconvolution showing more CD8 memory cells in the responders, while more CD4 memory cells and macrophages in the non-responders.

been clinically evaluated in HCC patients. This study represents a novel clinical approach by combining poly-ICLC with anti-PD1 for the treatment of unresectable HCC.

A hallmark of ISV is the abscopal effect, in which local radiotherapy induces antitumor responses at distant tumor sites. This phenomenon is thought to be mediated by circulating cytotoxic T lymphocytes (CTLs) and cytokines such as type I interferons, IL-6, IL-1 $\alpha$ , tumor necrosis factor- $\alpha$ , reactive oxygen species, and nitric oxide.<sup>29</sup> In this study, it is intriguing that Patient 4 exhibited shrinkage of a non-injected tumor, while another tumor remained unaffected following treatment. This differential response is unlikely to be due to the bystander effect, given the physical distance between the tumors. A more plausible explanation is intertumoral heterogeneity, which may have allowed the non-responsive tumor to evade recognition or destruction by tumor-specific CTLs.

Both intertumoral and intratumoral heterogeneity<sup>16,30,31</sup> are common in HCC and pose significant challenges for treatment strategies, including targeted therapies, ICIs, and neoantigen-based vaccines.<sup>32,33</sup> As an intratumoral agent, poly-ICLC offers the flexibility to be administered to multiple tumor sites during the course of treatment, potentially

addressing intertumoral heterogeneity. Furthermore, its ability to reach various regions within a single large HCC lesion may also help overcome intratumoral heterogeneity.

Both Patient 1 and Patient 4 presented with PVT and underlying cirrhosis. In cirrhotic livers, bland PVT is not uncommon and may result from disrupted hemostasis and damage to the portal vein due to portal hypertension and bacterial translocation.<sup>34</sup> In the context of HCC, however, PVT may represent PVTT, which is associated with poor prognosis.<sup>35,36</sup> Differentiating bland PVT from PVTT is critical in cirrhotic patients with HCC. A post-contrast increase in CT attenuation of  $\geq 20$  HU is indicative of PVTT.<sup>24,25</sup> At baseline, both patients were diagnosed with PVTT, but following treatment, Patient 1's PVT appeared to transition to a bland thrombus, while Patient 4's remained consistent with PVTT. This suggests that Patient 1's PVT may have initially contained both bland and tumorous components, with the tumor component resolving post-treatment. This interpretation is supported by a substantial decline in serum AFP levels—from 2,490 ng/mL to 2.11 ng/mL—and the disappearance of intrahepatic lesions, meeting the criteria for a CR (Table 1). Notably, ICI treatment has been associated with a 52.9% objective response rate in PVTT.<sup>37</sup>

In this study, both responders had baseline AFP levels  $\geq 400$  ng/mL, whereas the two non-responders had levels  $\leq 400$  ng/mL (Table 1). In the IMbrave150 study, patients with baseline AFP  $\leq 400$  ng/mL had better overall and progression-free survival following treatment with atezolizumab plus bevacizumab.<sup>38</sup> In contrast, the HIMALAYA<sup>39</sup> and CheckMate 459 studies<sup>40</sup> trials found that patients with AFP  $\geq 400$  ng/mL had improved survival following treatment with tremelimumab plus durvalumab and nivolumab, respectively. The results from our study are consistent with the latter findings. Albumin levels have been suggested as a prognostic biomarker in advanced cancer patients receiving ICIs.<sup>41</sup> However, since all patients in our study had normal pre-treatment albumin levels, its utility in predicting treatment response could not be assessed.

As demonstrated by Patient 1, the spatial transcriptome technique is a powerful tool for investigating gene expression profiles in HCC, particularly in needle biopsy samples from infiltrative HCCs that display intertwined normal parenchyma and cancerous tissue. This approach enables precise isolation of tumor RNAs (Figure 2). Transcriptome analysis revealed that IFN- $\gamma$  signaling was the most enriched pathway in the responders (Figure 5a). This finding aligns with a recent report highlighting the upregulation of IFN- $\gamma$  signaling and major histocompatibility complex (MHC) II-related antigen presentation as a molecular marker of response to anti-PD1 therapy in advanced HCC.<sup>42</sup> Notably, IFI30, one of the differentially expressed genes in responders (Figure 5b), is inducible by IFN- $\gamma$  and plays a role in both MHC I-restricted antigen cross-presentation and MHC II-restricted antigen processing by decreasing the disulfide bonds of endocytosed proteins.<sup>43</sup> Most tumor cells broadly express MHC-I, presenting tumor antigens that enable recognition and elimination by tumor-specific CD8<sup>+</sup> T cells.<sup>44</sup> However, a subset of tumors also expresses MHC-II, a molecule typically found on APCs. Recent studies have shown that MHC-II expression in tumor cells is linked to favorable outcomes and enhanced responses to immunotherapies.<sup>45</sup> Intriguingly, in this study, CD8 memory cells in TME were more abundant in responders, whereas CD4 memory cells were more in non-responders (Figure 5c). The presence of resident CD8 memory (CD8 T<sub>RM</sub>) cells in tumor infiltration is correlated with better prognosis and improved response to current immunotherapies.<sup>46</sup> In contrast, CD4 T<sub>RM</sub> cells are less well studied and characterized.<sup>47</sup> This study did not differentiate between helper and regulatory T cells, raising a question of whether the HCC tissues from non-responders contained a higher proportion of regulatory CD4 T<sub>RM</sub> cells. Along with CD4 memory cells, macrophages were more abundant in the TME of non-responders (Figure 5c). Macrophages are a double-edged sword in the TME, depending on their M1 and M2 phenotypes. Recently, they have emerged as a key target for developing new cancer immunotherapies.<sup>48,49</sup>

This study has several limitations. First, the small sample size constrains the statistical power and generalizability of the findings. Second, the absence of a control arm precludes definitive attribution of observed effects to the investigational therapy. Third, all enrolled patients were over 70 years old, and those with decompensated liver cirrhosis were excluded. This selective inclusion criterion may impact the treatment's efficacy, as older patients may respond differently to therapy, and excluding patients with more severe liver dysfunction limits the applicability of the results to a broader HCC population. Due to its premature termination, this study may pivot to exploring the combination of poly-ICLC with atezolizumab and bevacizumab in the future. To enhance the applicability, future studies should include a larger cohort encompassing a wider range of age groups and disease stages.

## Conclusions

Although limited by the small sample size, our preliminary findings suggest that the combination therapy of poly-ICLC and nivolumab may be a potentially safe and effective option for patients with unresectable HCC. Further studies involving larger patient cohorts and exploring additional combinations, such as atezolizumab plus bevacizumab, are warranted to confirm and expand upon these observations.

## Abbreviations

AE, Adverse events; CR, Complete response; dLNs, Draining lymph nodes; HCC, Hepatocellular carcinoma; ICIs, Immune checkpoint inhibitors; IM, Intramuscular; ISV, In situ vaccination; IT, Intratumoral; Mrecist, Modified Response Evaluation Criteria in Solid Tumors; ORR, Objective response rate; PD, Progressive disease; poly-ICLC, Polyinosinic–polycytidylic acid–poly-L-lysine carboxymethylcellulose; PR, Partial response; PVT, Portal vein thrombosis; TLR, Toll-like receptor.

## Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

## Ethics Approval and Informed Consent

The study protocol was approved by the Research Ethics Committee of the National Taiwan University Hospital (approval number: 202104104MIND) and performed in accordance with the ethical standards as laid down in the Helsinki Declaration of 1975, as revised in 2008. All participants signed informed consent for the study. This study was registered at ClinicalTrials.gov (identifier: NCT05281926).

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

Andres M. Salazar is the Chief Strategy Officer of Oncovir, Inc. In addition, Dr Andres M. Salazar has patents 7439349, 7834064, 8592391, EP 1778186 issued to Oncovir; patents 18/445640 & 18/445641 pending to Oncovir. The other authors have no conflicts of interest to declare for this work.

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