Supplemental material

Intratumoral Bicarbonate Functions as an Adjuvant to Potentiate PD-1 Blockade in Hepatocellular Carcinoma

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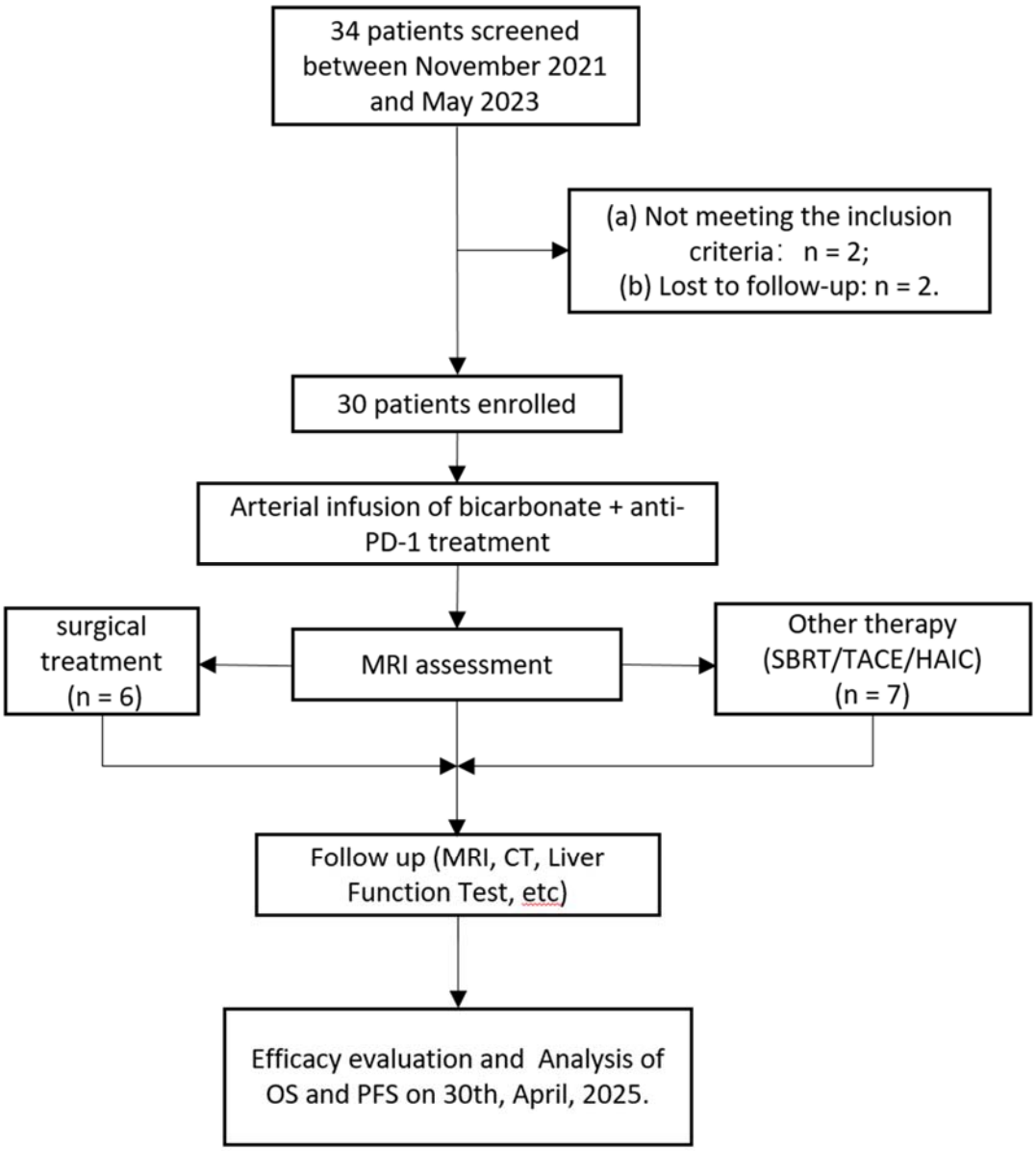

Figure S1. Trial flow diagram.

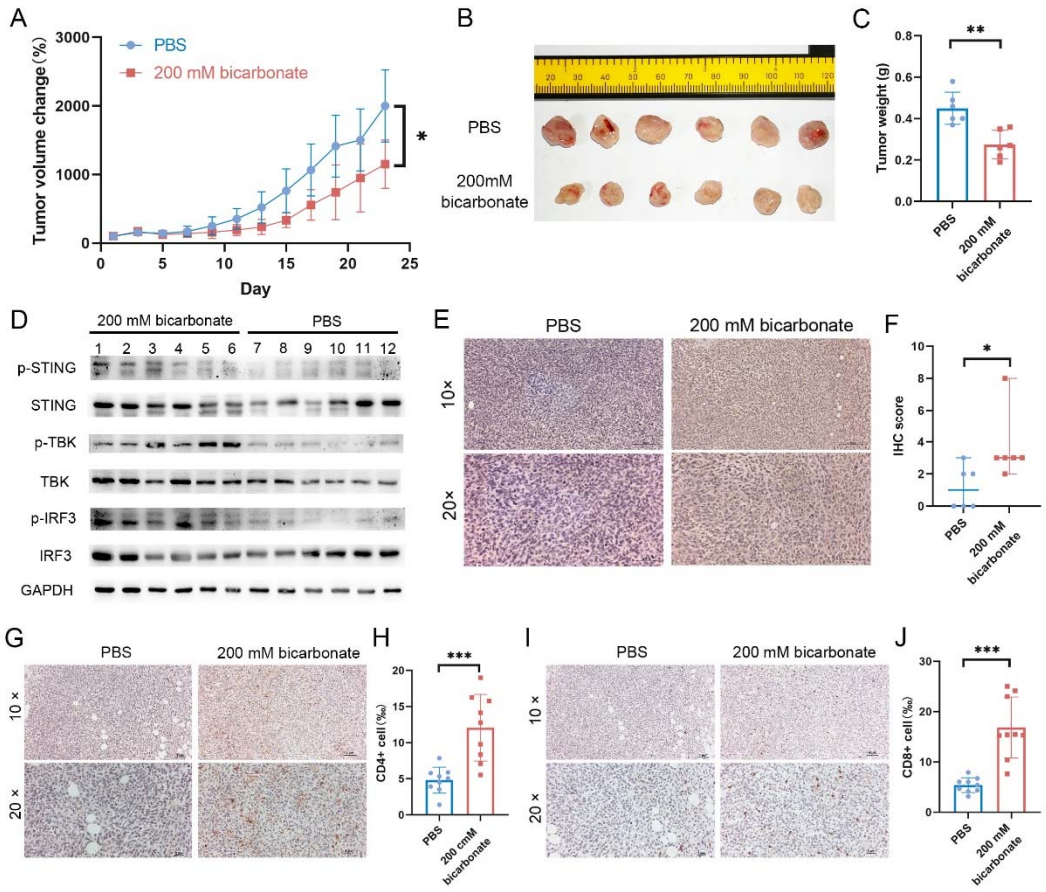

Figure S2. Bicarbonate inhibits tumor growth. Twelve BALB/c mice were orthotopically inoculated with 4T1 cells in the mammary fat pad. When tumor size reached 4 to 6 mm in the largest diameter, the mice were divided into two groups and received intratumoral injections of either PBS or 200 mM bicarbonate every other day. (A) Tumor volume of 4T1 orthotopic tumors. (B) Tumors were dissected and photographed after 12 injections. (C) Tumor weight. (D) Phosphorylation levels of STING/TBK1/IRF3 in tumor tissues. (E) Representative IHC image showing IFN-α expression in tumor tissues. (F) IHC scoring of IFN-α in tumor tissues. (G) Representative IHC image showing $CD4^+$ cells in tumor tissues. (H) Percentage of $CD4^+$ cells in tumor tissues. (I) Representative IHC image showing $CD8^+$ cells in tumor tissues. (J) Percentage of $CD8^+$ cells in tumor tissues. Data are presented as mean ± SD (n = 6). Statistical analyses: two-way ANOVA for (A), Mann-Whitney U test for (F), and two-tailed Student's t-tests for the other comparisons. *p < 0.05, **p < 0.01, ***p < 0.001.

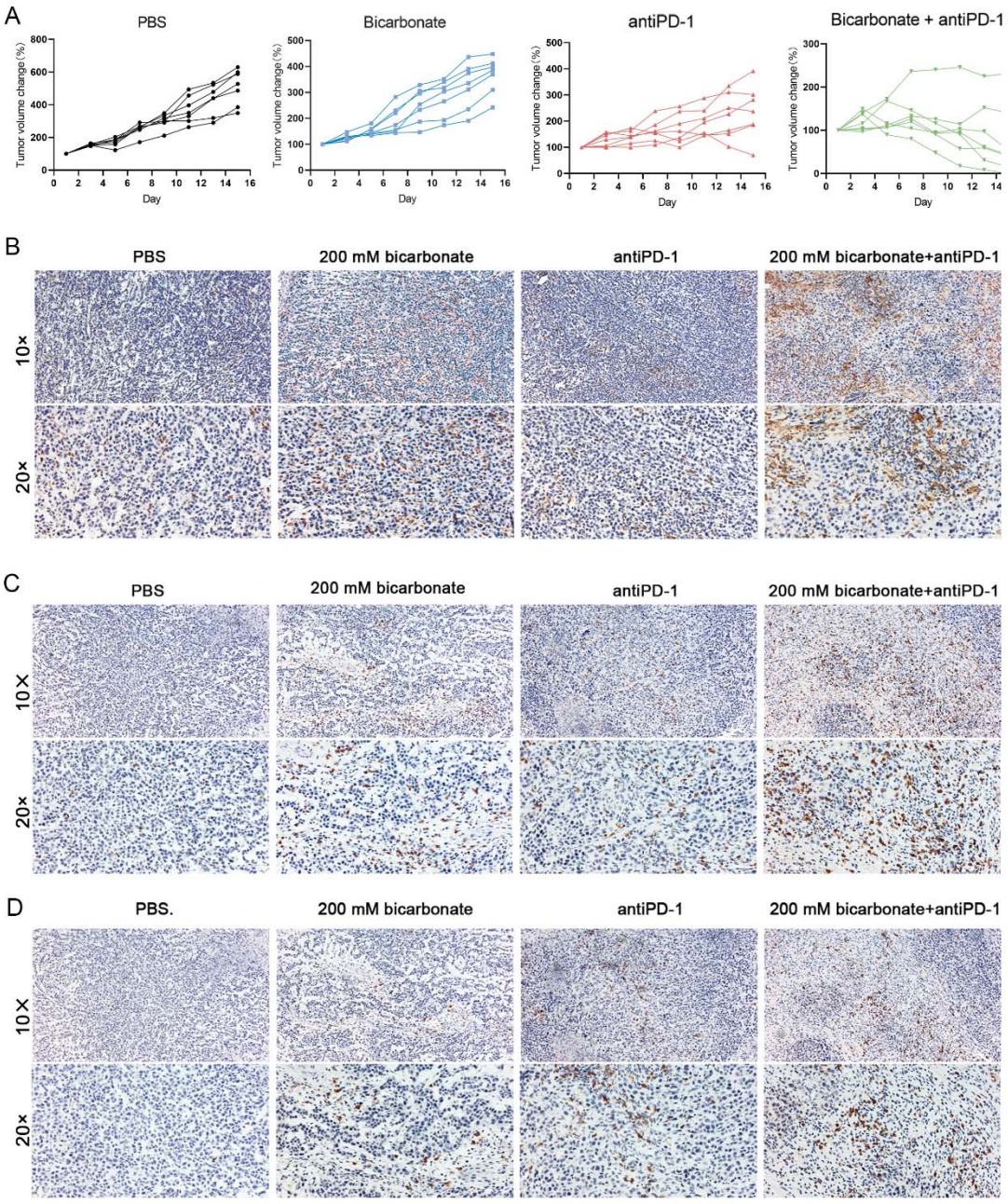

Figure S3. Intratumoral Injection of bicarbonate Combined with AntiPD-1 Therapy for Breast Cancer. (A) Individual tumor volume changes in each mouse. (B)Representative IHC images of $CD11c^+$ cells in breast tumor tissues. (C)Representative IHC images of $CD8^+$ cells in breast tumor tissues. (D) Representative IHC images of $CD4^+$ cells in breast tumor tissues.

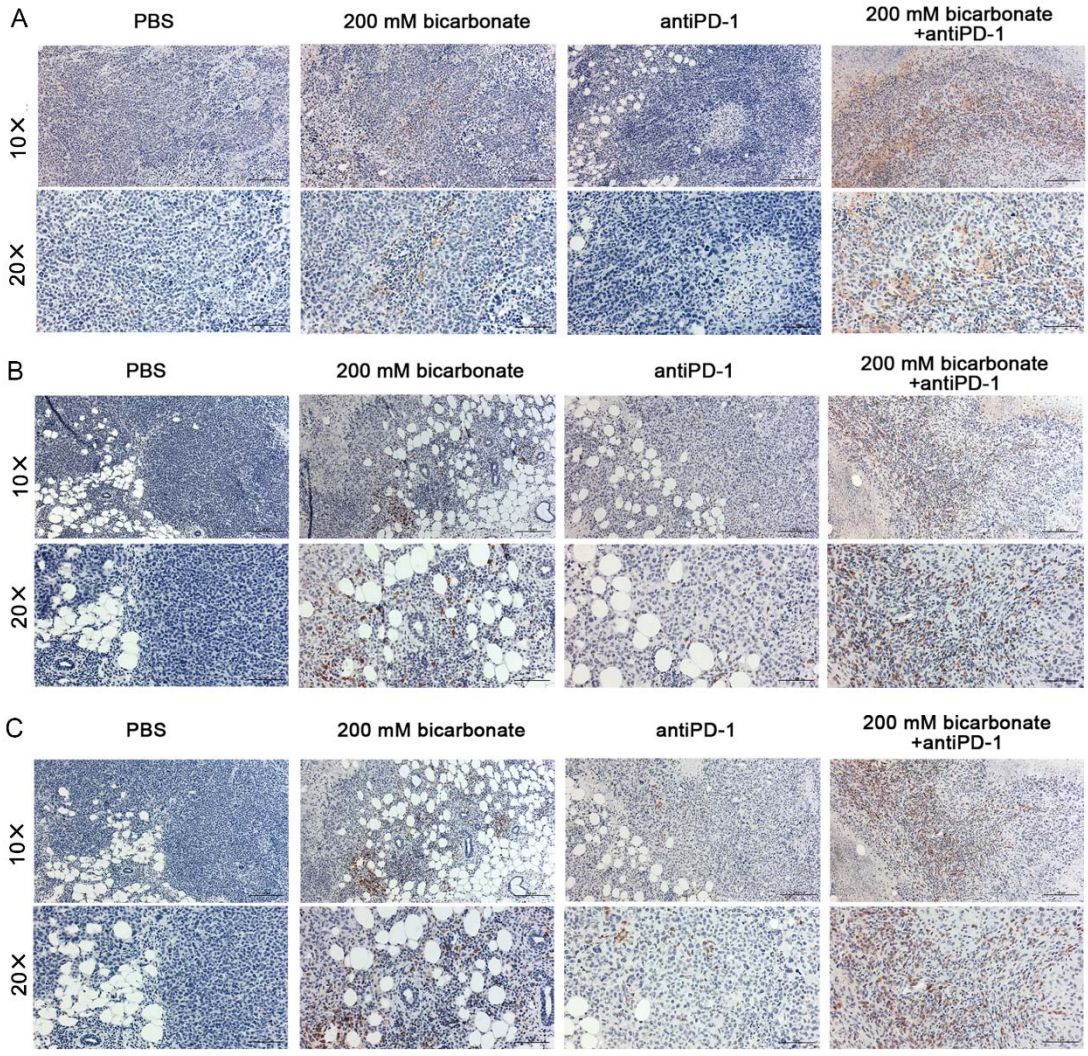

Figure S4. Intratumoral Injection of bicarbonate Combined with AntiPD-1 Therapy for HCC.

(A) Representative IHC images of $CD11c^+$ cells in breast tumor tissues. (B)Representative IHC images of $CD8^+$ cells in breast tumor tissues. (C) Representative IHC images of $CD4^+$ cells in breast tumor tissues.

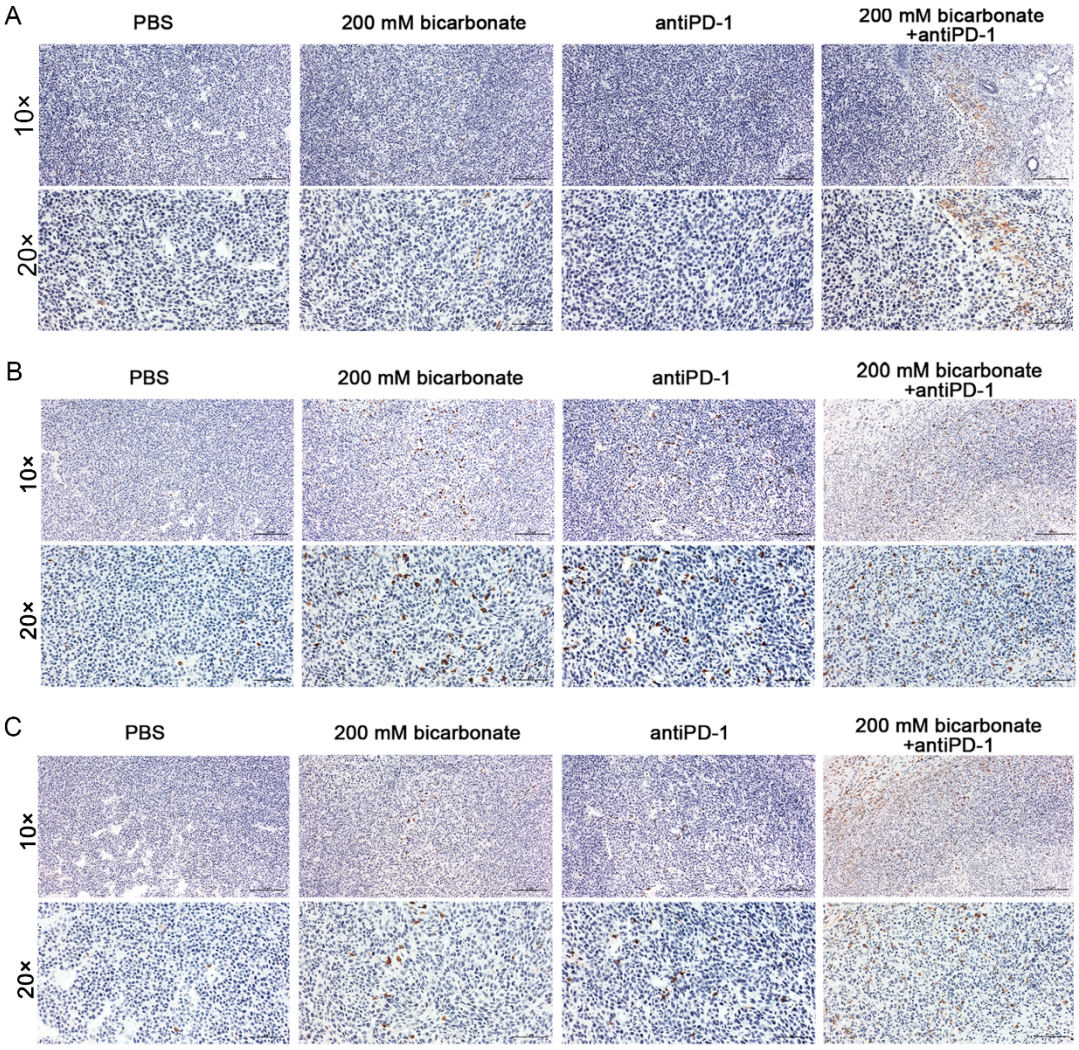

Figure S5. Intratumoral Injection of bicarbonate Combined with AntiPD-1 Therapy for CRC. (A) Representative IHC images of $CD11c^+$ cells in breast tumor tissues. (B)Representative IHC images of $CD8^+$ cells in breast tumor tissues. (C) Representative IHC images of $CD4^+$ cells in breast tumor tissues.

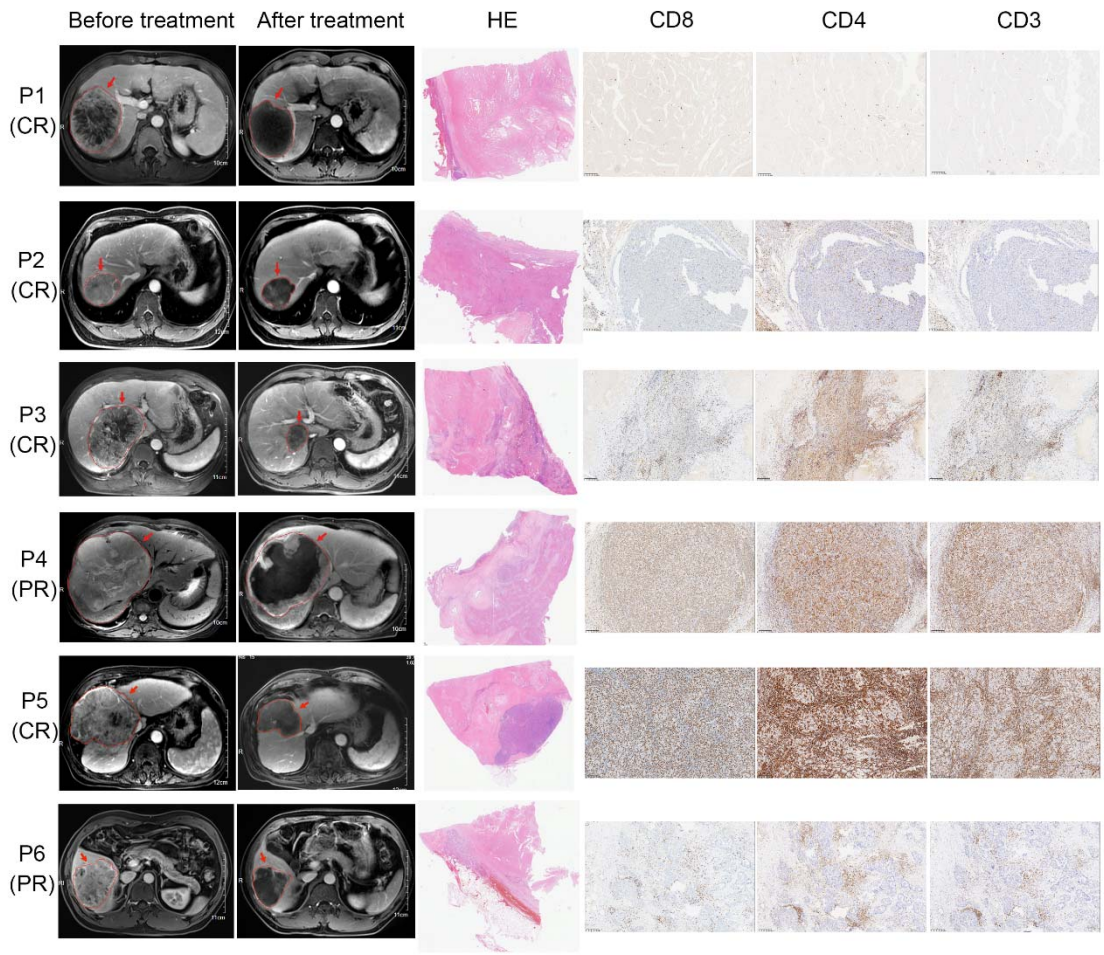

Figure S6. The treatment response, MRI images, and pathological slices of patients (P1-P6) in the bicarbonate + antiPD-1 group. For each patient, the images are arranged fro left to right: before treatment MRI image, after treatment MRI image, HE staining image, CD8 IHC image, CD4 IHC image, and CD3 IHC image. In the MRI images, the red circle indicated by the arrow marks the tumor region.

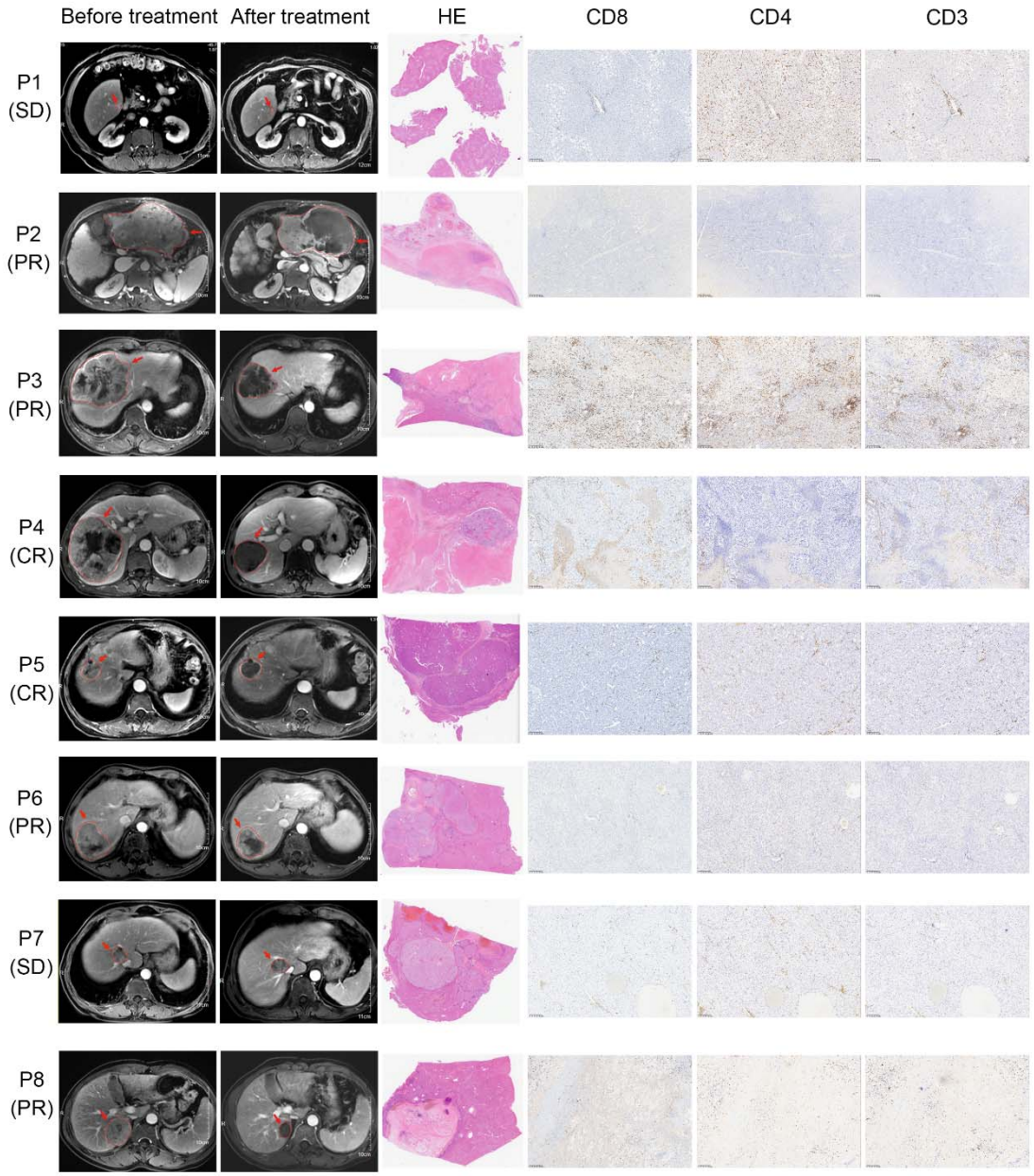

Figure S7. The treatment response, MRI images, and pathological slices of patients in the antiPD-1 group. 8 patients (P1-P8), with each patient's images arranged from left to right as follows: before treatment MRI image, after treatment MRI image, HE staining image, CD8 IHC image, CD4 IHC image, and CD3 IHC image. In the MRI images, the red circle indicated by the arrow marks the tumor region.

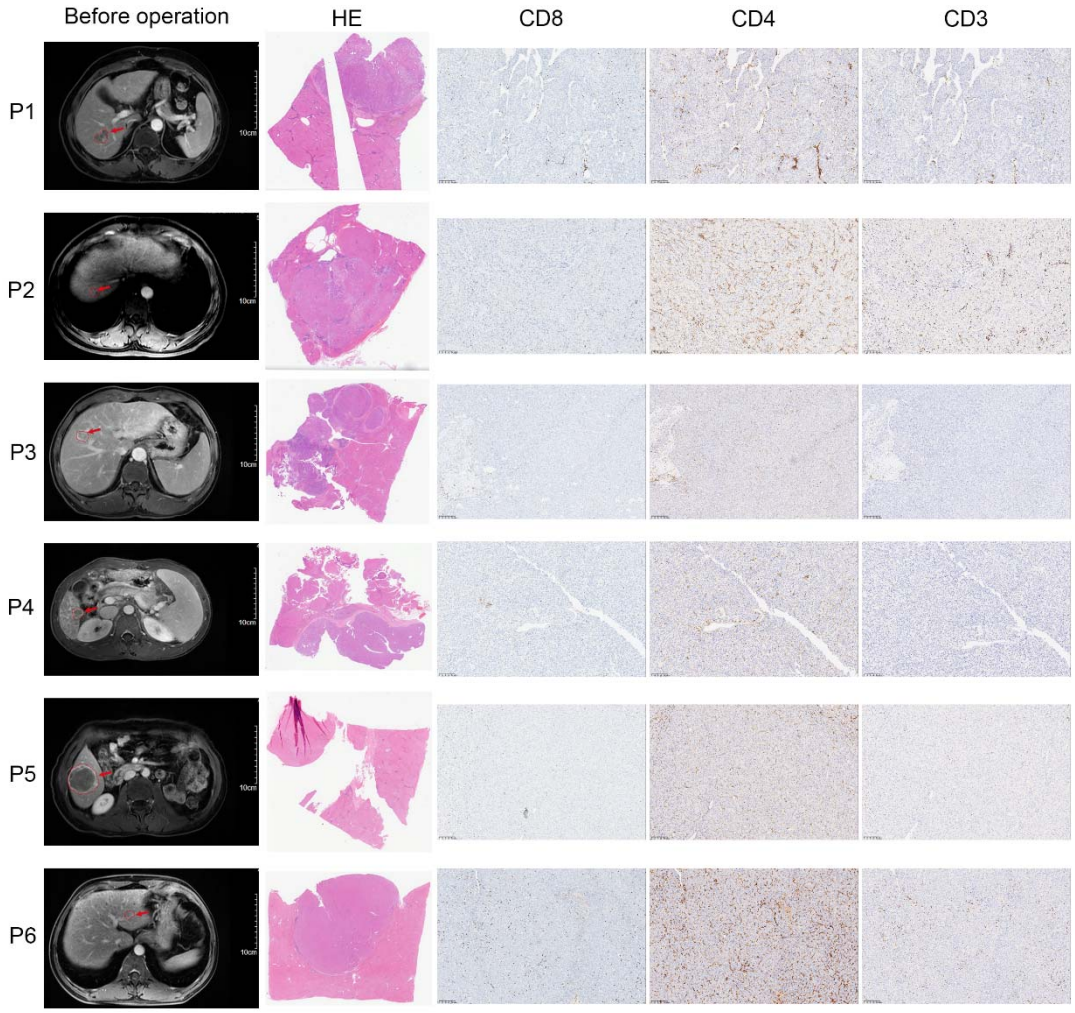

Figure S8. The MRI images and pathological slices of patients (P1-P6) in the surgery group. Before operation MRI images, HE staining image, CD8 IHC image, CD4 IHC image, and CD3 IHC image of surgery group patients (P1-P6). The red circle indicating the tumor region is marked by an arrow in the MRI images.

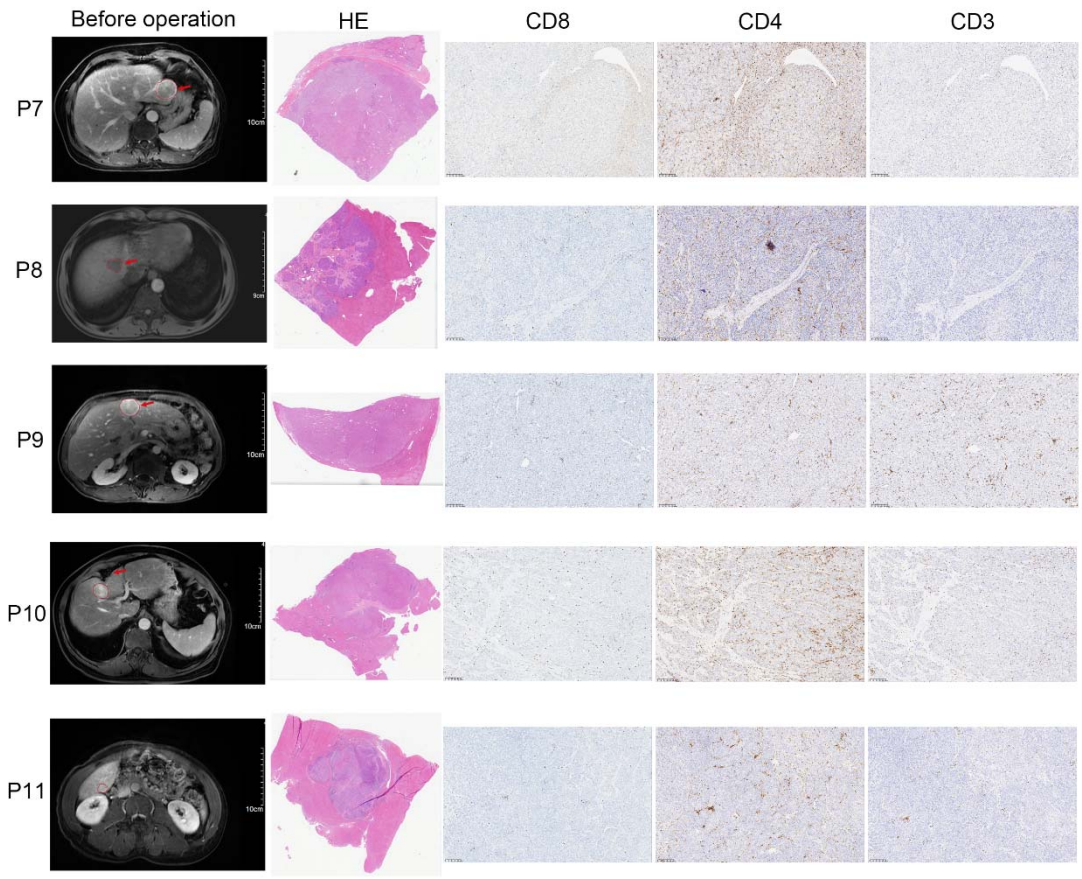

Figure S9. The MRI images and pathological slices of patients (P7-P11) in the surgery group. Before operation MRI images, HE staining image, CD8 IHC image, CD4 IHC image, and CD3 IHC image of surgery group patients (P7-P11). The red circle indicating the tumor region is marked by an arrow in the MRI images.

Table S1. Primers

Primers	Sequences
Homo-GAPDH-F	AGATCCCTCCAAAATCAAGTGG
Homo-GAPDH-R	GGCAGAGATGATGACCCTTTT
Homo-ND1-F	CTCTTCGTCTGATCCGTCCT
Homo-ND1-R	TGAGGTTGAGGTCTGTTAGT
Homo-ND2-F	GTAGACAGTCCCACCCTCAC
Homo-ND2-R	TTGATCCCGTTTCGTGCAAG
Homo-IFN-β-F	ATGACCAACAAGTGTCTCCTCC
Homo-IFN-β-R	GGAATCCAAGCAAGTTGTAGCTC
Homo-IFN-α-F	GCTTGGGATGAGACCCTCCTA
Homo-IFN-α-R	CCCACCCCCTGTATCACAC

Table S2. Antibodies

Target	Catalog number	Brand	RRID
Calreticulin	27298-1-AP	Proteintech (China)	AB_2880835
HMGB1	10829-1-AP	Proteintech (China)	AB_2232989
STING	19851-1-AP	Proteintech (China)	AB_10665370
Phospho-STING (Ser366)	50907	CST (USA)	AB_2827656
Phospho-STING (Ser365)	72971	CST (USA)	AB_2799831
IRF3	11312-1-AP	Proteintech (China)	AB_2127004
IRF3	4302	CST (USA)	AB_1904036
Phospho-IRF-3 (Ser396)	4947	CST (USA)	AB_823547
TBK1/NKA	3504	CST (USA)	AB_2255663
Phospho-TBK1/NAK (Ser172)	5483	CST (USA)	AB_10693472
dsDNA	ab27156	Abcam (USA)	AB_470907
GAPDH	2118	CST (USA)	AB_561053
PD-1	BE0146	bio X cell (USA)	AB_10949053
CD11c	97585	CST (USA)	AB_2800282
CD8	ab217344	Abcam (USA)	AB_2890649
CD4	ab183685	Abcam (USA)	AB_2686917
CD3	17617-1-AP	Proteintech (China)	AB_1939430
IFN-α2	ab193055	Abcam (USA)	AB_3076700

Table S3. The characteristics of patients

Variables	Hepatic arterial infusion of bicarbonate + antiPD-1
Cases	30
Gender (M/F)	23/7
Median age, years	59 (range 26 - 85)
HBV	27 (90.0%)
AST, U/L	70.8±45.5
ALT, U/L	46.5±26.5
AFP > 400 ng/mL	20 (66.7%)
Bilirubin, μmol/L	19.6±8.1
Albumin, g/L	39.2±4.9
Child-Pugh class	
A	29 (96.7%)
B	1 (3.3%)
The size of largest tumor:	11.4 (range 5.6 – 16.3)
Tumor diameter ≤ 5 cm	0
5 cm < Tumor diameter ≤ 10 cm	11 (36.7%)
Tumor diameter >10 cm	19 (63.3%)
Number of intrahepatic lesions	
1	16 (53.3%)
≥2	14 (46.7%)
BCLC stage:	
0	0
A	0
B	2 (6.7%)
C	28 (93.3%)
Macrovascular invasion:	28 (93.3%)
Only PV invasion	14 (46.7%)
Only PVTT	8 (26.7%)
Only HV invasion	5 (16.7%)
Only HVTT	3 (10.0%)
PV + HV invasion	9 (30.0%)
PVTT + HVTT	2 (6.7%)
Extra-hepatic metastasis	4 (13.3%)

HBV, hepatitis B virius;

AST, Aspartate transaminase;

ALT, Alanine aminotransferase;

AFP, alpha-feto-protein;

BCLC，Barcelona Clinic Liver Cancer;

PV, Portal vein;

HV, Hepatic vein;

PVTT, Portal vein tumor thrombus;

Table S4. Tumor response

Characteristic	N = 30[1]	95% CI
tumor response		
CR	16 (53.3%)	35%, 71%
PR	12 (40.0%)	23%, 59%
SD	2 (6.7%)	1.2%, 24%

[1]n (%)

Abbreviation: CI = Confidence Interval

Table S5. mOS, the estimated 1-year, 2-year and 3-year overall survival rates

Characteristic	1-year (95% CI)	2-year (95% CI)	3-year (95% CI)	mOS (95% CI)	P value[1]
Overall	82% (69%, 98%)	60% (44%, 81%)	53% (36%, 78%)	— (16, —)	
Age					0.17
< 60 years	79% (61%, 100%)	51% (30%, 85%)	40% (21%, 80%)	31 (15, —)	
≥ 60 years	86% (69%, 100%)	70% (49%, 100%)	— (—, —)	— (16, —)	
Tumor size[2]					0.76
> 10 cm	83% (67%, 100%)	57% (38%, 87%)	57% (38%, 87%)	— (16, —)	
≤ 10 cm	82% (62%, 100%)	64% (41%, 99%)	— (—, —)	31 (16, —)	
Tumor count					0.00024
multi	62% (41%, 95%)	27% (10%, 69%)	13% (2.5%, 72%)	16 (12, —)	
single	100% (100%, 100%)	87% (71%, 100%)	87% (71%, 100%)	— (—, —)	
AFP>400 ng/mL					0.012
No	100% (100%, 100%)	90% (73%, 100%)	90% (73%, 100%)	— (—, —)	
Yes	73% (55%, 96%)	42% (24%, 74%)	35% (18%, 68%)	16 (13, —)	
Tumor thrombus					0.47
No	87% (72%, 100%)	67% (47%, 95%)	57% (36%, 91%)	— (16, —)	
Yes	77% (57%, 100%)	51% (30%, 89%)	51% (30%, 89%)	— (13, —)	

[1]Log-rank test

[2]Diameter of largest tumor

Table S6. mPFS，the estimated 1-year and 2-year PFS rates

Characteristic	1-year (95% CI)	2-year (95% CI)	mPFS (95% CI)	P value[1]
Overoll	55% (38%, 80%)	55% (38%, 80%)	31 (9.0, —)	
Age				0.23
< 60 years	53% (30%, 94%)	53% (30%, 94%)	31 (3.0, —)	
≥ 60 years	59% (37%, 95%)	59% (37%, 95%)	— (10, —)	
Tumor size[2]				0.24
> 10 cm	43% (23%, 79%)	43% (23%, 79%)	12 (5.0, —)	
≤ 10 cm	76% (52%, 100%)	76% (52%, 100%)	31 (31, —)	
Tumor count				0.0024
multi	28% (11%, 71%)	28% (11%, 71%)	8.0 (3.0, —)	
single	83% (63%, 100%)	83% (63%, 100%)	— (—, —)	
Tumor thrombus				0.55
No	56% (34%, 93%)	56% (34%, 93%)	31 (3.0, —)	
Yes	56% (33%, 94%)	56% (33%, 94%)	— (9.0, —)	
AFP>400 ng/mL				0.0035
No	100% (100%, 100%)	100% (100%, 100%)	— (—, —)	
Yes	35% (17%, 69%)	35% (17%, 69%)	9.0 (5.0, —)	

[1]Log-rank test

[2]Diameter of largest tumor

Table S7. Adverse events

Adverse events	Total	CTCAE ≥ grade 3		
		Grade 3	Grade 4	Grade 5
Fever	30	-	-	-
Abdominal pain	7 (23.3%)	-	-	-
Nausea	3 (10.0%)	-	-	-
Vomit	1 (3.3%)	-	-	-
Bone marrow				
Immumotherapy-related toxicity	4 (13.33%)	-	-	-
Immumotherapy-related pneumonia	0	-	-	-
Immumotherapy-related hypothyroidism	3 (10.0%)	-	-	-
Skin ulcer/rash/erythema	3 (10.0%)	-	-	-
Liver enzyme abnormalities	25 (83.3%)	-	-	-
Bilirubin-related abnormalities	9 (30.0%)	-	-	-
Liver abscess	0	-	-	-
Cholecystitis	0	-	-	-

CTCAE，the Common Terminology Criteria for Adverse Events.