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Supplementary Figure Legends

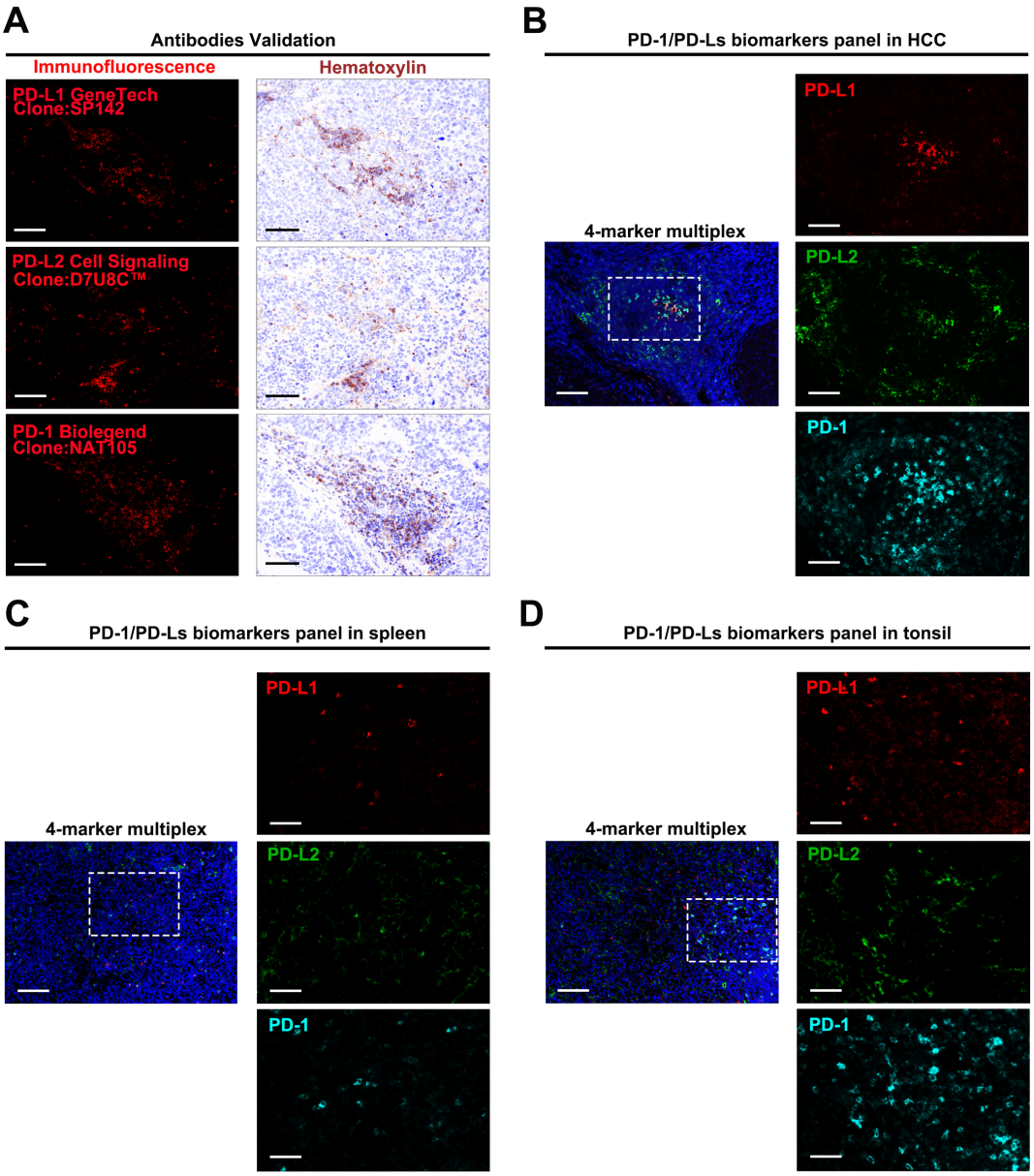
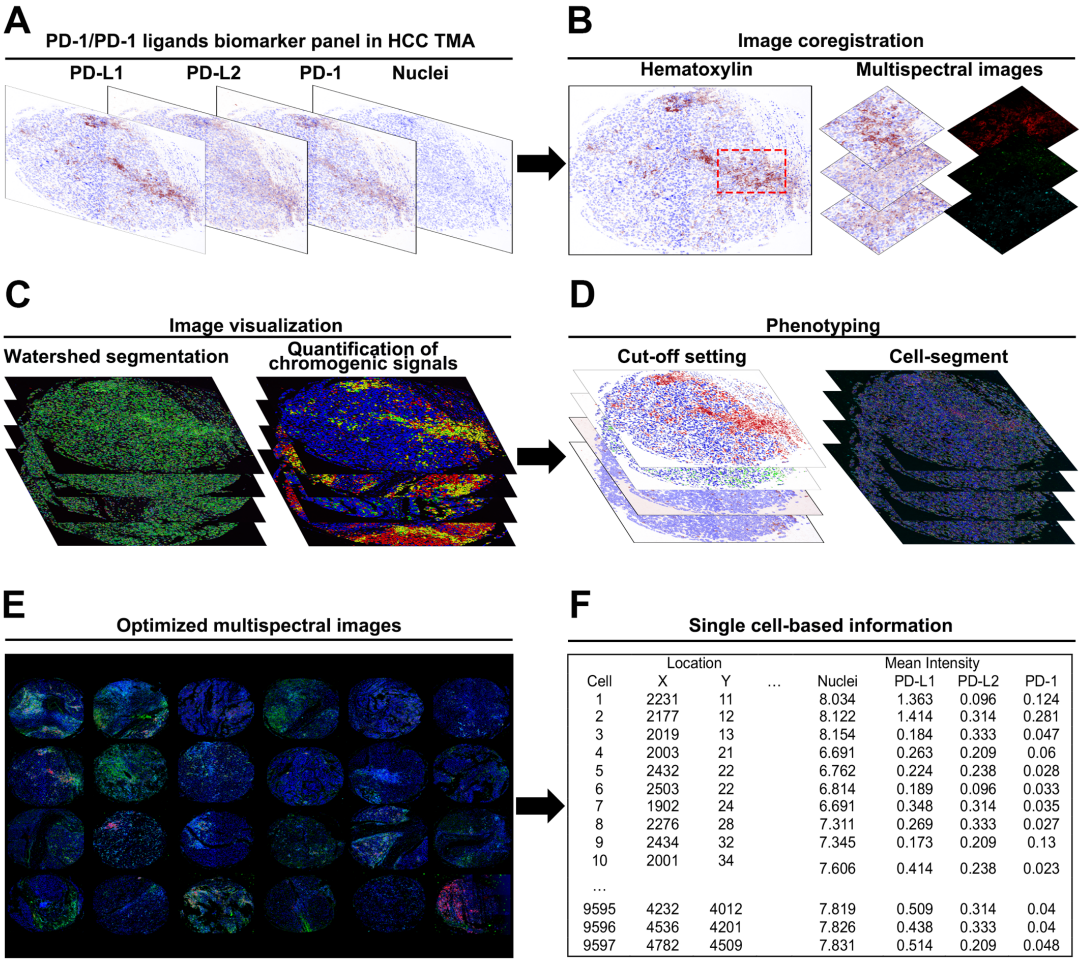


Figure S1. Optimization of multispectral immunofluorescence staining for PD-L1/PD-L2/PD-1.

(A) The optimization and validation of PD-L1/PD-L2/PD-1 antibodies used in full HCC FFPE slides. PD-1 (clone NAT105, Biolegend), PD-L1 (clone SP142, Genetech) and PD-L2 (clone D7U8C™, Cell Signaling Technology) were chosen to apply in following experiments. **(B)** Representative fluorescence images displayed each of the three biomarker respectively in HCC tumor tissue. **(C)** Spleen and **(D)** tonsil were used as positive controls. Scale bar=100 μm.



	Location			Mean Intensity			
Cell	X	Y	…	Nuclei	PD-L1	PD-L2	PD-1
1	2231	11		8.034	1.363	0.096	0.124
2	2177	12		8.122	1.414	0.314	0.281
3	2019	13		8.154	0.184	0.333	0.047
4	2003	21		6.691	0.263	0.209	0.06
5	2432	22		6.762	0.224	0.238	0.028
6	2503	22		6.814	0.189	0.096	0.033
7	1902	24		6.691	0.348	0.314	0.035
8	2276	28		7.311	0.269	0.333	0.027
9	2434	32		7.345	0.173	0.209	0.13
10	2001	34		7.606	0.414	0.238	0.023
…							
9595	4232	4012		7.819	0.509	0.314	0.04
9596	4536	4201		7.826	0.438	0.333	0.04
9597	4782	4509		7.831	0.514	0.209	0.048

Figure S2. Schematic overview of four-colors multispectral immunofluorescence work flow in HCC.

(A) Representative images in bright field displayed the each of the three biomarker antibodies respectively and staining sequence in TMA. **(B-D)** Sequential steps of multiplexing work flow for analysis procedures. **(B)** Hematoxylin-stained images used for image coregistration, each individual marker in the composite image after spectral unmixing was highlighted in the enlarged subsection of the core as shown. **(C)** Image visualization, including watershed segmentation and quantification of chromogenic signals. **(D)** Image phenotyping, including setting up of optimized cut-off value and segment of individual cells. **(E)** The TMA core map consisted of representative composited images were shown in tumor and peritumor tissues respectively. **(F)** Acquisition of single cell information, pixel intensities of chromogenic signals were extracted and recorded by single-cell analysis, together with location in original images.

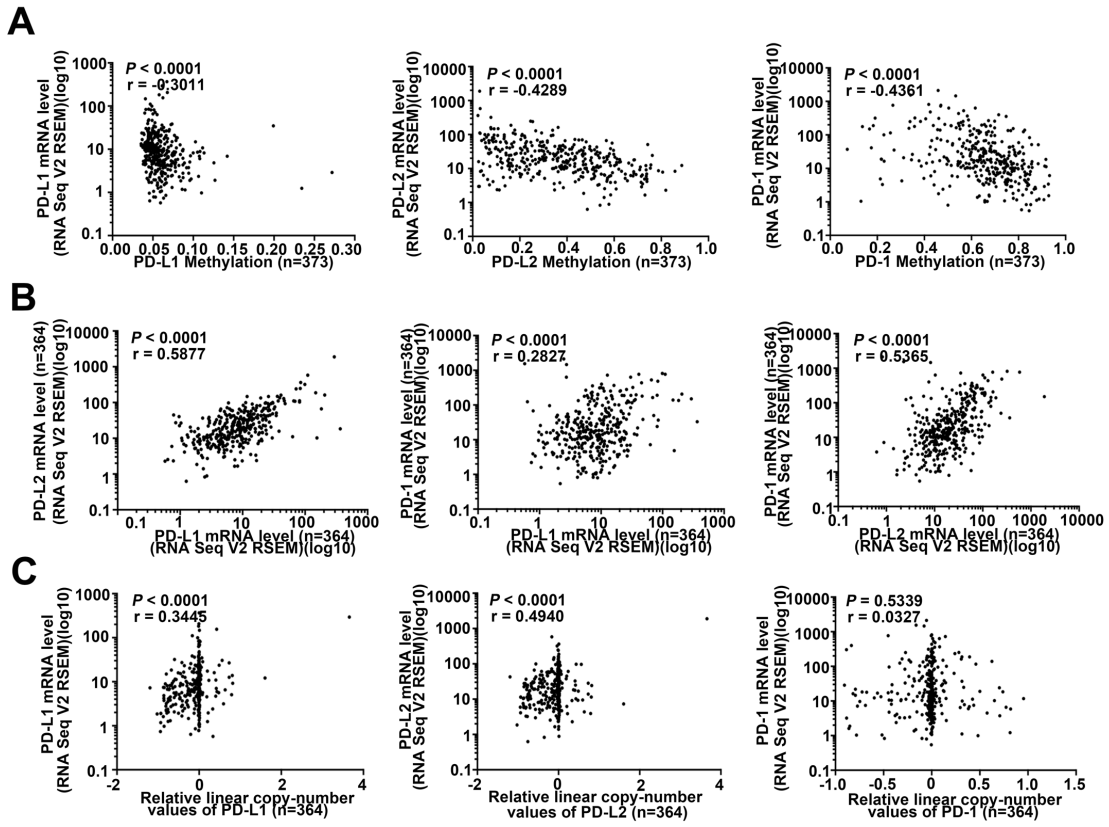


Figure S3. Copy number values and transcriptional levels of PD-L1/PD-L2/PD-1 evaluated in HCC patients from TCGA database.

(A) Significantly negative correlations were observed between PD-Ls/PD-1 DNA methylation and mRNA expression (n = 373). **(B)** Markedly positive correlations were observed among the mRNA expression levels of PD-1 and PD-Ls. **(C)** Significantly positive correlations were found between copy number values and mRNA expression of PD-L1/PD-L2, but not in PD-1. Pearson correlation analysis.

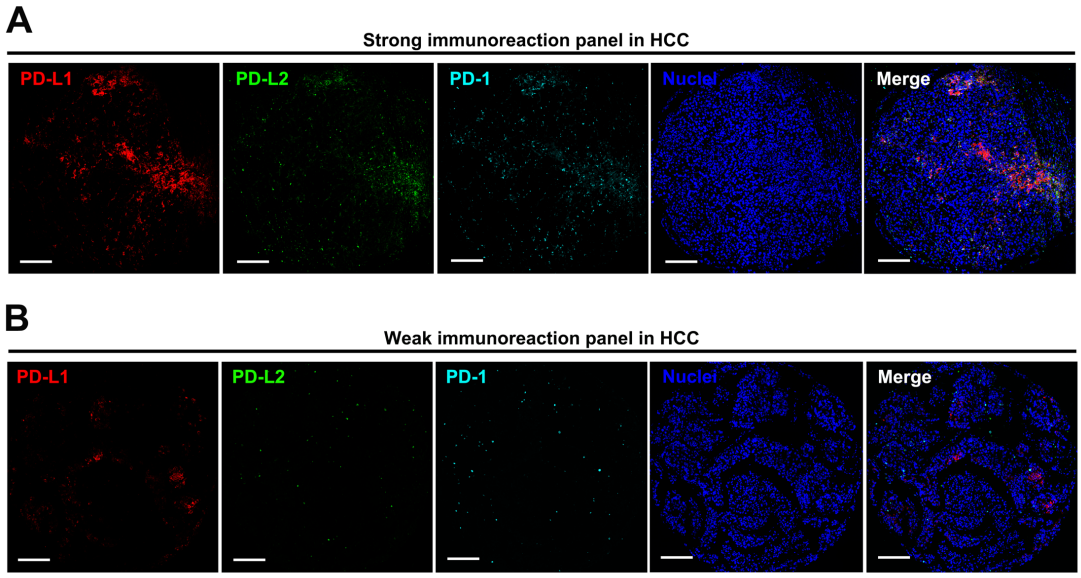


Figure S4. Representative immunofluorescence images displayed two types of PD-Ls/PD-1 expression status in HCC.

(A) Strong immunoreaction was observed with up-regulated PD-Ls expression and high-dense PD-1^{+} immunocytes infiltration in HCC tumor tissues. **(B)** Weak immunoreaction was uncovered with limited PD-Ls expression and low-dense PD-1^{+} immunocytes infiltration in HCC tumor tissues. Scale bar=50 μm.

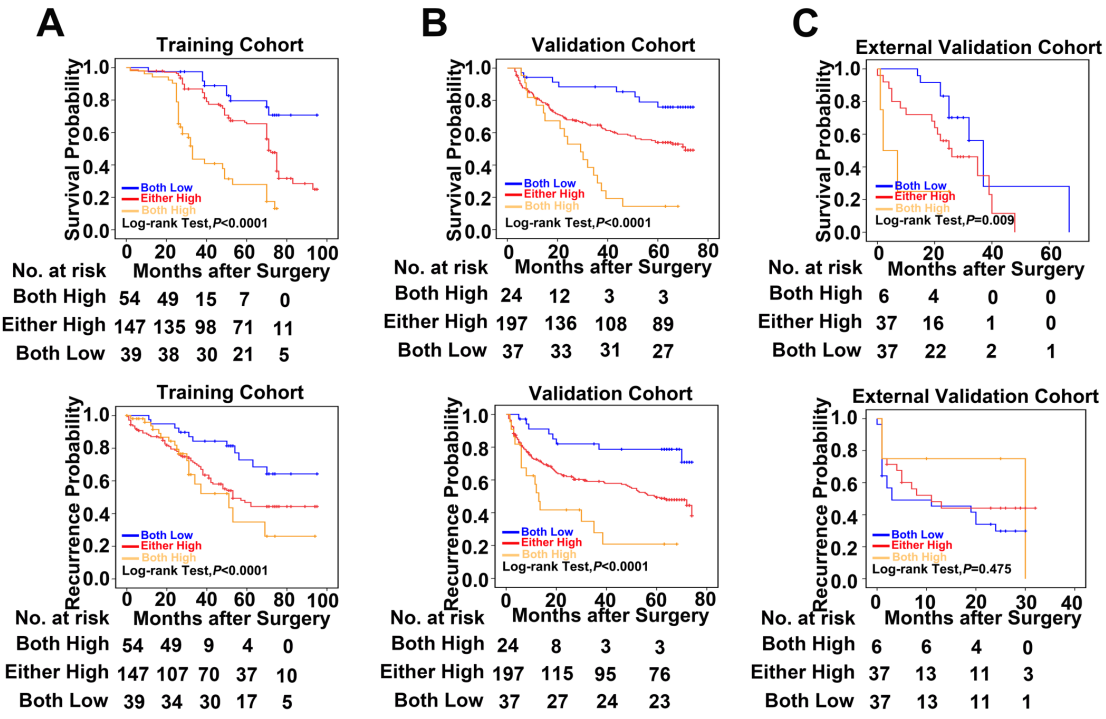


Figure S5. Prognostic value of combination of PD-L1 expression and 9p24.1 alterations.

(A) Kaplan-Meier curves showing that OS and TTR for the combination of PD-L1 expression

and 9p24.1 alteration were found to be statistically significant in training cohort (n = 240). **(B)** Significant differences in OS and TTR were validated in validation cohort based on combination of PD-L1 expression and 9p24.1 alteration (n = 258). **(C)** Significant difference in OS was validated in external validation cohort based on combination of PD-L1 expression and 9p24.1 alteration (n = 80). *P* values was determined by the log-rank test.

Table S1. Patients clinical and pathologic characteristics.

Characteristics	Training cohort (n=240)	Validation cohort (n=258)	External independent cohort (n=80)	*P**
Age (years) (Median, Range)	56(18-83)	49(10-79)	44(29-75)	0.051
Gender (Male vs. Female)	192/48	220/38	51/29	0.635
Virus infection (Yes vs. No)	222/18	255/3	77/3	0.786
Preoperative α-Fetoprotein (ng/ml) (Median, Range)	85.3(0-60500.0)	6671.8(0-60500.0)	1137(0.9-60500.0)	0.904
Preoperative alanine aminotransferase (U/L) (Median, Range)	35(8-851)	36(10-561)	NA	0.377†
Preoperative g-glutamyl transferase (units/L) (Median, Range)	57(10-632)	63(13-648)	NA	0.013†
Preoperative total bilirubin (μmol/L) (Median, Range)	11.4(2.2-240.3)	13.3(4.4-37.1)	15.2(6.3-76.7)	0.323
Liver cirrhosis (Yes vs. No)	202/38	233/25	62/18	0.702
Maximum diameter (cm) (≤5 vs. >5)	139/101	137/121	59/21	0.052
Tumor encapsulation (Complete vs. None)	146/94	144/114	65/15	0.118
Tumor multiplicity (multiple vs. single)	56/184	45/213	60/20	0.623
Tumor differentiation (poor vs. well)	80/160	77/181	31/49	0.329

Vascular invasion (Yes vs. No)	87/153	91/167	29/51	0.212
Hepatic hilar lymph node metastasis (Yes vs. No)	8/232	6/252	NA	0.421†
UICC TNM stage (I vs. II-III)	161/79	146/112	28/52	0.169
BCLC stage (0-A vs. B-C)	103/137	102/156	18/62	0.116
Alive with of recurrence (Yes vs. No)	75/165	121/137	36/44	0.168

Abbreviation: UICC, International Union Against Cancer; TNM, Topography Lymph Node Metastasis; BCLC: Barcelona Clinic Liver Cancer. *The Chi square test was applied. †Unpaired t test was applied.

Table S2. Univariate analysis of factors associated with recurrence and survival in three independent cohorts (n=578).

	OS			TTR		
	Training cohort	**Validation cohort**	**External cohort**	**Training cohort**	**Validation cohort**	**External cohort**
Variables	**Univariate *P****	**Univariate *P****	**Univariate *P****	**Univariate *P****	**Univariate *P****	**Univariate *P****
Age, years (>51 vs. ≤51)	0.889	0.957	0.156	0.852	0.727	0.340
Gender (male vs. female)	0.973	0.490	0.258	0.476	0.843	0.445
HBsAg (positive vs. negative)	0.155	0.319	0.167	0.321	0.410	0.867
HBcAg (positive vs. negative)	0.743	0.295	NA	0.681	0.211	NA
Serum AFP, ng/ml (>20 vs. ≤20)	0.247	**0.001**	0.241	0.959	**0.012**	0.230
Serum ALT, U/L (>75 vs. ≤75)	0.843	0.344	NA	0.613	0.279	NA
Serum γ-GT, U/L (>54 vs. ≤54)	**<0.0001**	**0.015**	NA	**0.040**	0.057	NA
Liver cirrhosis (yes vs. no)	0.728	0.473	0.887	0.299	0.859	0.775
Tumor size (cm) (>5 vs. ≤5)	**<0.0001**	**<0.0001**	0.191	**0.004**	**<0.0001**	0.140
Tumor multiplicity (multiple vs. single)	**<0.0001**	0.229	0.297	0.336	0.072	0.769
Tumor differentiation (poor vs. well)	**<0.0001**	**<0.0001**	0.725	**0.012**	**0.012**	0.094
Tumor encapsulation (yes vs. no)	**0.012**	**0.045**	0.073	**0.002**	**0.034**	0.476
Vascular invasion (yes vs. no)	**<0.0001**	**<0.0001**	0.318	0.374	**<0.0001**	0.673

TNM stage (III-II vs. I)	**0.006**	**<0.0001**	0.191	**0.030**	**<0.0001**	0.140
BCLC stage (B-C vs. 0-A)	**0.012**	**<0.0001**	0.436	**0.001**	**<0.0001**	0.089
9p24.1 alteration (with vs. without)	**<0.0001**	**0.0009**	**0.019**	0.392	**0.024**	0.843
mRNA level of PD-L1 (High vs. Low)	0.598	NA	NA	0.556	NA	NA
mRNA level of PD-L2 (High vs. Low)	0.437	NA	NA	0.913	NA	NA
PD-L1 tumor (High vs. Low)	**<0.0001**	**0.003**	**0.024**	**0.005**	**0.008**	0.548
PD-L2 tumor (High vs. Low)	**0.007**	**0.002**	**0.002**	0.138	**0.002**	0.893

NOTE: *Kaplan–Meier method (log-rank test) in SPSS was performed to accomplish univariate analysis.

Abbreviations: TTR, time to recurrence; HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B center antigen; AFP, alpha-fetoprotein; ALT, alanine transaminase; γ-GT, γ-glutamyltransferase; TNM, tumor-node-metastasis; BCLC, Barcelona Clinic Liver Cancer.

Table S3. Multivariate analysis of factors associated with OS and TTR.

Variables	OS						TTR					
	Training cohort			Validation cohort			Training cohort			Validation cohort		
	HR	95%CI	*P**	HR	95%CI	*P**	HR	95%CI	*P**	HR	95%CI	*P**
Serum AFP, ng/ml (>20 vs. ≤20)	NA	NA	NA	1.46	0.87-2.44	0.150	NA	NA	NA	**1.46**	**0.97-2.20**	**0.068**
Serum γ-GT, U/L (>54 vs. ≤54)	**1.64**	**1.11-2.40**	**0.011**	1.00	0.49-2.04	0.994	1.20	0.78-1.86	0.404	NA	NA	NA
Tumor size (cm) (>5 vs. ≤5)	**1.91**	**1.29-2.82**	**0.001**	1.52	0.91-2.55	0.106	**1.59**	**1.01-2.48**	**0.044**	1.53	0.93-2.53	0.098
Tumor multiplicity (multiple vs. single)	1.14	0.74-1.76	0.542	NA	NA	NA	NA	NA	NA	NA	NA	NA
Tumor differentiation (poor vs. well)	**1.56**	**1.05-2.32**	**0.026**	**1.55**	**1.05-2.29**	**0.027**	**0.01**	**1.16-2.79**	**0.009**	**1.55**	**1.05-2.28**	**0.026**
Tumor encapsulation (yes vs. no)	0.78	0.53-1.13	0.192	1.04	0.70-1.53	0.853	**0.62**	**0.41-0.95**	**0.027**	1.04	0.70-1.53	0.853
Vascular invasion (yes vs. no)	**1.57**	**1.05-2.36**	**0.028**	1.41	0.89-2.23	0.148	NA	NA	NA	1.41	0.89-2.22	0.142
TNM stage (III-II vs. I)	1.56	0.98-2.49	0.058	**1.65**	**1.02-2.65**	**0.041**	0.95	0.56-1.59	0.835	1.65	1.03-2.63	0.037
BCLC stage (B-C vs. 0-A)	0.95	0.59-1.53	0.857	**3.09**	**1.59-6.05**	**0.001**	**1.86**	**1.06-3.26**	**0.030**	**3.10**	**1.59-6.02**	**0.001**
PD-L1 tumor (High vs. Low)	**2.11**	**1.44-3.08**	**<0.001**	**2.16**	**1.38-3.38**	**0.001**	**1.78**	**1.17-2.71**	**0.007**	**2.16**	**1.38-3.38**	**0.001**

NOTE: * Cox proportional hazards models in SPSS was performed to accomplish multivariate analysis.

Abbreviations: OS, overall survival; TTR, time to recurrence; AFP, alpha-fetoprotein; γ-GT, γ-glutamyltransferase; TNM, tumor-node-metastasis; BCLC, Barcelona Clinic Liver Cancer; NA, not applicable.

Table S4. Multivariate analysis of factors associated with OS and TTR.

	OS						TTR					
	Training cohort			Validation cohort			Training cohort			Validation cohort		
Characteristics	**HR**	**95%CI**	***P****	**HR**	**95%CI**	***P****	**HR**	**95%CI**	***P****	**HR**	**95%CI**	***P****
Serum AFP, ng/ml (>20 vs. ≤20)	NA	NA	NA	1.58	0.95-2.65	0.080	NA	NA	NA	**1.55**	**1.03-2.34**	**0.035**
Serum γ-GT, U/L (>54 vs. ≤54)	**1.66**	**1.13-2.42**	**0.009**	0.96	0.47-1.95	0.900	1.23	0.80-1.90	0.341	NA	NA	NA
Tumor size (cm) (>5 vs. ≤5)	**1.91**	**1.28-2.86**	**0.001**	1.40	0.84-2.34	0.198	1.52	0.97-2.38	0.068	1.39	0.84-2.29	0.196
Tumor multiplicity (multiple vs. single)	1.12	0.73-1.73	0.611	NA	NA	NA	NA	NA	NA	NA	NA	NA
Tumor differentiation (poor vs. well)	1.38	0.93-2.05	0.113	**1.63**	**1.11-2.41**	**0.013**	**1.80**	**1.16-2.80**	**0.009**	**1.63**	**1.11-2.39**	**0.013**
Tumor encapsulation (yes vs. no)	0.71	0.49-1.03	0.067	1.14	0.77-1.68	0.527	**0.57**	**0.38-0.87**	**0.008**	1.13	0.77-1.68	0.528
Vascular invasion (yes vs. no)	**1.53**	**1.03-2.28**	**0.036**	1.45	0.93-2.27	0.106	NA	NA	NA	1.44	0.93-2.26	0.106
TNM stage (III-II vs. I)	**1.70**	**1.06-2.74**	**0.029**	1.36	0.86-2.17	0.191	0.94	0.56-1.59	0.821	1.37	0.87-2.16	0.178
BCLC stage (B-C vs. 0-A)	0.85	0.52-1.38	0.505	**3.09**	**1.59-6.00**	**0.001**	**1.76**	**1.00-3.10**	**0.050**	**3.10**	**1.59-6.01**	**0.001**
PD-L2 tumor (High vs. Low)	1.50	0.96-2.35	0.079	1.44	0.98-2.11	0.065	NA	NA	NA	1.44	0.98-2.11	0.065

NOTE: *Cox proportional hazards models in SPSS was performed to accomplish multivariate analysis.

Abbreviations: OS, overall survival; TTR, time to recurrence; AFP, alpha-fetoprotein; γ-GT, γ-glutamyltransferase; TNM, tumor-node-metastasis; BCLC, Barcelona Clinic Liver Cancer; NA, not applicable.

Table S5. Multivariate analysis of factors associated with survival and recurrence.

	Training cohort						Validation cohort					
	Multivariate						Multivariate					
	OS			TTR			OS			TTR		
Variables	**HR**	**95%CI**	***P****	**HR**	**95%CI**	***P****	**HR**	**95%CI**	***P****	**HR**	**95%CI**	***P****
Serum AFP, ng/ml (>20 vs. ≤20)		NA			NA		**1.70**	**1.03-2.28**	**.038**	1.36	0.92-2.01	0.118
Serum γ-GT, U/L (>54 vs. ≤54)	**1.55**	**1.05-2.28**	**0.027**	1.27	0.82-1.97	0.291	0.85	0.42-1.73	0.653		NA	
Tumor size (cm) (>5 vs. ≤5)	**1.86**	**1.25-2.77**	**0.002**	1.52	0.97-2.37	0.068	**1.68**	**1.03-2.72**	**0.037**	1.16	0.74-1.83	0.525
Tumor differentiation (poor vs. well)	1.33	0.89-1.98	0.163	**1.79**	**1.15-2.80**	**0.010**	**1.64**	**1.11-2.43**	**0.013**	1.17	0.78-1.75	0.450
Tumor encapsulation (yes vs. no)	0.79	0.54-1.15	0.214	**0.63**	**0.42-0.96**	**0.032**	1.16	0.79-1.70	0.457	1.21	0.83-1.76	0.330
Vascular invasion (yes vs. no)	**1.59**	**1.06-2.38**	**0.026**		NA		**1.70**	**1.10-2.63**	**0.017**	**1.75**	**1.11-2.76**	**0.016**
TNM stage (III-II vs. I)	1.55	0.98-2.43	0.061	0.93	0.55-1.55	0.768		NA			NA	
BCLC stage (B-C vs. 0-A)	0.89	0.55-1.44	0.626	1.71	0.97-2.99	0.062	**2.69**	**1.36-5.34**	**0.004**	**2.09**	**1.13-3.87**	**0.019**
Combined PD-L1 and 9p24.1 alterations#		NA	**<0.001**		NA	0.123		NA	**0.004**		NA	**0.003**
I vs. II	**0.38**	**0.25-0.58**	**<0.001**	0.49	0.23-1.06	0.069	0.59	0.35-1.00	0.052	0.66	0.37-1.16	0.148
I vs. III	**0.18**	**0.08-0.38**	**<0.001**	0.94	0.54-1.64	0.827	**0.23**	**0.10-0.55**	**0.001**	**0.22**	**0.09-0.54**	**0.001**

NOTE: *Cox proportional hazards regression model, including all the clinic-pathologic features as covariates.

[#]Patients were divided into three groups: (I) both high expression of PD-L1 and 9p24.1 amplification/polysomy, (II) either high expression of PD-L1 or 9p24.1 amplification/polysomy, and (III) both low expression of PD-L1 and 9p24.1 disomy.

Abbreviations: OS, overall survival; TTR, time to recurrence; AFP, alpha-fetoprotein; γ-GT, γ-glutamyltransferase; TNM, tumor-node-metastasis; BCLC, Barcelona Clinic Liver Cancer; NA, not applicable.

Table S6. Correlations of clinicopathologic characteristics with PD1/PD-Ls and PD-1 in HCC patients from training cohort (n=240) and validation cohort (n=258).

Characteristics	Training cohort (n=240)									Validation cohort (n=258)								
	PD-L1			PD-L2			PD-1			PD-L1			PD-L2			PD-1		
	High	Low	*P**	High	Low	*P**	High	Low	*P**	High	Low	*P**	High	Low	*P**	High	Low	*P**
Age, years																		
≤51	42	51	0.382	64	29	0.750	78	69	0.586	87	44	0.527	61	70	0.262	81	50	0.261
> 51	58	89		104	43		46	47		89	38		68	59		87	40	
Gender																		
Female	22	26	0.513	31	17	0.359	25	23	0.948	24	14	0.468	15	23	0.159	22	16	0.318
Male	78	114		137	55		99	93		152	68		114	106		146	74	
HBsAg																		
Negative	4	14	0.082†	13	5	0.831	7	11	0.259	5	4	0.406†	4	5	0.734†	7	2	0.417†

Positive	96	126		155	67		117	105		171	78		125	124		161	88	
HBcAb																		
Negative	10	24	0.118	23	11	0.747	14	20	0.186	5	4	0.406†	5	4	0.734†	8	1	0.127†
Positive	90	116		145	61		110	96		171	78		124	125		160	89	
AFP (ng/ml)																		
≤20	38	53	**0.022**	59	32	0.172	44	47	0.422	60	39	**0.038**	47	52	0.522	61	38	0.352
>20	62	87		109	40		80	69		116	43		82	77		107	52	
ALT (U/L)																		
≤75	82	120	0.437	141	61	0.877	105	97	0.823	152	74	0.379	117	109	0.131	145	81	0.391
>75	18	20		27	11		19	19		24	8		12	20		23	9	
γ-GT (U/L)																		
≤54	50	74	0.662	82	42	0.176	61	63	0.428	34	17	0.791	28	23	0.434	30	21	0.293
>54	50	66		86	30		63	53		142	65		101	106		138	69	

Liver cirrhosis																	
No	18	20	0.437	22	16	0.076	21	17	0.628	20	11	0.637	13	18	0.338	15	16	**0.037**
Yes	82	120		146	56		103	99		156	71		116	111		153	74	
Tumor size (cm)																	
≤5	50	89	**0.036**	88	51	**0.008**	60	79	**0.002**	77	42	0.262	48	71	**0.004**	75	44	0.514
>5	50	51		80	21		64	37		99	40		81	58		93	46	
Tumor number																	
Single	78	106	0.680	131	53	0.464	96	88	0.776	147	61	0.084	103	105	0.753	132	76	0.255
Multiple	22	34		37	19		28	28		29	21		26	24		36	14	
Vascular invasion																	
No	66	87	0.540	103	50	0.229	77	76	0.582	108	55	0.376	73	90	**0.028**	98	65	**0.028**
Yes	34	53		65	22		47	40		68	27		56	39		70	25	
Tumor																	

None	46	48	0.067	69	25	0.356	53	41	0.241	74	36	0.779	58	52	0.450	66	44	0.137
Complete	54	92		99	47		71	75		102	46		71	77		102	46	
Tumor																		
I+II	63	97	0.309	101	59	**0.001**	86	74	0.361	116	60	0.244	90	86	0.593	112	64	0.357
III+IV	37	43		67	13		38	42		60	22		39	43		56	26	
TNM stage																		
I	59	102	**0.024**	99	62	**<0.001**	71	90	**<0.001**	142	57	**0.047**	93	106	0.054	123	76	**0.041**
II+III	41	38		69	10		53	26		34	25		36	23		45	14	
BCLC stage																		
0-A	35	68	**0.036**	78	25	0.093	47	56	0.105	62	39	**0.043**	37	60	**0.003**	60	53	**<0.001**
B-C	65	72		90	47		77	60		118	43		92	69		108	37	

NOTE: *The Pearson Chi square test was applied. † Chi-square with Yates' correction was applied.

Abbreviations: HCC, hepatocellular carcinoma; HBsAg, hepatitis B surface antigen; HBcAb, hepatitis B center antibody; AFP, alpha-fetoprotein; ALT, alanine transaminase; γ-GT, γ-glutamyltransferase; TNM, tumor-node-metastasis; BCLC, Barcelona Clinic Liver Cancer.