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ORIGINAL RESEARCH

Establishment of Nomogram Model for Minimally Invasive Treatment of Small Hepatocellular Carcinoma Based on CD8$^+$T Cell Counts

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Purpose: Minimally invasive treatment of small hepatocellular carcinoma (HCC) is the main way of treatment, which can cause the change of HCC immune microenvironment. T lymphocytes are an important part of the immune microenvironment and may be powerful predictors of prognosis. The purpose of this study was to explore the effect of T lymphocytes on the prognosis of HCC and establish a prognostic model.

Patients and Methods: We conducted a retrospective study of 300 patients with small HCC and developed a clinical prediction model. The selection of modeling variables was performed by combining backward stepwise Cox regression using Akaike's Information Criteria (AIC) and the Least Absolute Shrinkage and Selection Operator (LASSO) regression. Establish a dynamic nomogram model to predict 1-, 2-, and 3-year overall survival (OS). Receiver operating characteristic curve (ROC curve) was used to verify the model discriminative ability, calibration curve was used to examine the model calibration ability, and decision curve analysis (DCA) was used to evaluate the clinical value.

Results: The nomogram to predict the OS of small HCC includes the following four variables: aspartate aminotransferase (AST), alpha fetoprotein (AFP), C-reactive protein (CRP) and CD8$^+$T cell counts, represented liver function index, tumor-related index, Inflammatory index and immune-related index, respectively. The area under the receiver operating characteristic curves (AUC) of predicting 1-, 2-, and 3-year overall survival were 0.846, 0.824 and 0.812, and the model was excellent in discrimination, calibration and clinical applicability.

Conclusion: Our study provides a nomogram based on CD8$^+$T cell counts that can help predict the prognosis of small HCC after minimally invasive treatment, which suggests that T lymphocytes can be used as a prognostic factor for HCC. Larger trials are needed to verify our results.

Keywords: small hepatocellular carcinoma, nomogram, minimally invasive treatment, CD8$^+$T cell counts, overall survival

Introduction

According to global Cancer Statistics 2020, Hepatocellular carcinoma (HCC) is the sixth most frequent cancer in the world, ranking fifth in incidence and third in mortality.[1] The worldwide incident of HCC is estimated to exceed one million by 2025.[2] HCC is a major public health problem. When most patients are diagnosed with HCC, they are already in the middle and late stages with a very high mortality rate. Although various advanced treatment measures are being actively explored and good progress has been made successively, the survival rate is still low.[3] Thus, early detection, early diagnosis and early treatment are essential to enhance the therapeutic effect of HCC.

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Small HCC (less than 3 cm in diameter) is the main type of early HCC. With the continuous progress of cancer surveillance technology, the detection rate of small HCC is increasing year by year. According to guidelines recommended, the main treatment methods for small HCC include surgical interventions (hepatectomy and liver transplantation) and minimally invasive treatments (radiofrequency ablation (RFA) and trans-arterial chemoembolization (TACE)).[4] Surgical intervention is the most effective potential cure for small HCC, the 5-year postoperative survival rate is 60%, and the postoperative mortality rate is low (< 3%).[5] Nonetheless, in clinical practice, due to the high cost of surgery and organ shortage, the number of patients who really benefit is limited. In contrast, minimally invasive treatment, as an equally effective option, is relatively easy to perform and has a wider range of clinical applications for patients with early-stage HCC who are not candidates for surgery.[6]

RFA is the most common local ablation technique, which has been recommended by the American Association for the Study of Liver Diseases (AASLD)[7] and the European Association for the Study of the Liver (EASL)[8] as the primary first-line treatment for single tumor < 2 cm and as an alternative surgical treatment in early-stage single tumor 3–4 cm or 2–3 tumours < 3 cm. The main principle of its operation is to achieve the purpose of anti-tumor by utilizing the needle electrode to generate an electric current, which locally induces thermocytotoxicity based on heat, thus leading to the thermal coagulation necrosis of the tumor.[9] TACE is also recommended in the guidelines by injecting chemical embolization agents into the artery to make the tumor ischemic necrosis.[10] It is usually used for unresectable HCC and combined with RFA in the treatment of local tumors with vascular passage. Due to the cooling effect of liver blood flow, RFA alone is mostly poor in this case, and TACE combined with RFA often achieve better results.

Although the clinical benefits of minimally invasive treatment are similar to those of surgical intervention, the risk of relapse and death cannot be ignored.[6] Studies have shown that two-thirds of patients who undergo minimally invasive treatment experience recurrence or develop new tumors.[11] At present, there are many classical HCC prediction models, including the Japan Integrated Staging (JIS) score, Okuda, the tumor node metastasis (TNM) staging, the Cancer of the Liver Italian Program (CLIP) score, the Chinese University Prognostic Index (CUPI), etc., but most of them are suitable for advanced HCC, even in the early stage, most of them were built for postoperative purposes.[12] Therefore, it is vital to establish a prognosis model suitable for small HCC after minimally invasive surgery. Many studies have reported that RFA can induce immune regulation in the body by activating innate immune response and adaptive immune response successively through local inflammatory response, and activating T lymphocytes response, including the increase of $CD8^+$T cells, memory $CD8^+$T cells, and the decrease of regulatory T (Treg) cells.[13] Meanwhile, TACE has been demonstrated to modulate the immune microenvironment by reducing the density of immune-exhausted effector cytotoxic and Treg cells.[14] In patients treated with TACE, the number of Treg cells in peripheral blood decreased significantly, and the proportion of $CD4^+$T cells and $CD4^+/CD8^+$T cells increased to varying degrees. Immunomarkers may be useful prognostic indicators for possible changes in the immune microenvironment after minimally invasive treatment.[15]

Previous studies of our team have found that NK cell counts can predict long-term survival of HCC, and has a better prediction effect for advanced HCC.[16] We further explored the prognostic indicators in the immune microenvironment of patients with early small HCC after minimally invasive treatment. With the in-depth study of tumor immune microenvironment, attention has been paid to the role of T lymphocytes, and they have gradually become routine clinical test indicators, which play a great role in inhibiting the occurrence and development of tumors and removing cancer cells, and can affect the recurrence and death of diseases.[17] T lymphocytes may be an important indicator of survival in patients with small HCC after minimally invasive treatment. However, a variety of current prognostic models mostly focus on common clinical indicators and imaging data,[18] suggesting that they do not pay attention to the possible predictive role of T lymphocytes in the prognosis of HCC.

The present study is to explore the effect of T lymphocytes on the prognosis of HCC, and to establish a prognostic model of small HCC by combining them with clinical indicators, providing help for the assessment and prevention of prognostic risk.

Materials and Methods

Patient Population and Data Collection

This is a retrospective, single-center cohort study involving 300 patients diagnosed with small HCC after minimally invasive treatment at Beijing Ditan Hospital, Capital Medical University from May 2016 to August 2018. Patients were randomly assigned to a 70% training cohort or a 30% validation cohort. This study was approved by the Ethics Committee of Beijing Ditan Hospital. The inclusion criteria for this study were: ① Patients diagnosed with HCC according to guidelines[8] (based on biopsy [histological or cytological examination], imaging, or alpha-fetoprotein [AFP level ≥ 400 ng/mL] serological results); ② Characteristics of HCC: maximum diameter ≤ 3 cm, no more than 3 nodules, no extrahepatic metastasis or tumor portal vein thrombosis; ③ Treatment was TACE or RAF or a combination of both. ④ Complete clinical data, including T lymphocytes. Exclusion criteria: ① Pregnant women and patients with serious diseases of other organs; ② Patients with incomplete clinical data; ③ Patients with vascular invasion and extrahepatic metastasis.

Baseline demographic and clinical examination data were collected, including: sex, age, personal history (drinking and smoking), past medical history (hypertension, coronary heart disease and diabetes), reasons, treatments, complications (hepatic encephalopathy, upper gastrointestinal hemorrhage (UGIB), ascites and portal hypertension), Child-Pugh stage, biochemical examination indexes (white blood cells (WBC), neutrophil counts, lymphocyte counts, neutrophil-lymphocytes ratio (NLR), albumin, alkaline phosphatase (ALP), platelet counts (PLT), International Normalized Ratio (INR), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), prothrombin time activity (PTA), alpha fetoprotein (AFP), and C-reactive protein (CRP)), and T lymphocytes (T cell counts, $CD8^+$T cell counts, $CD4^+$T cell counts and CD4/CD8). The primary outcome was overall survival (OS), defined as the time interval from the date of enrollment to the onset of death or the end of follow-up. The survival group was defined as having no death during follow-up, while the death group was defined as having died from any cause between enrollment and the end of follow-up. Patient survival was determined by telephone follow-up every 3 months and hospital record system.

In order to objectively evaluate the applicability of the new model, the discriminative power of the classical models (TNM, JIS, Okuda, CLIP, and CUPI) were obtained for comparative analysis. The main components of these classical models were as follows: the TNM score[19] (including: tumour size and spread to lymph nodes/metastases); the JIS score[20] (including: based on TNM score plus AFP and Child-Pugh stage); the Okuda score[21] (including: tumour size, ascites, albumin and TBIL); the CLIP score[22] (including: Child-Pugh stage, tumor morphology, AFP, and portal vein thrombosis); the CUPI score[23] (including: based on TNM score plus AFP, ascites, TBIL and ALP).

Statistical Analysis

In this study, all data were processed by R software (Version 4.1.2) and categorical variables. For some indicators that the original data were continuous variables, they were converted into categorical variables according to the normal range used by our hospital or the median of the indicator. The variables were described in the form of integer and proportion, and comparative analysis was conducted by χ^2test or Fisher's exact test. Based on clinical experience and published literature, indicators related to HCC survival rate were selected as candidate variables.[24] The candidate variables were analyzed by the following two modeling methods, when backward stepwise Cox regression was used to screen variables, the meaningful values ($p < 0.05$) after univariate analysis were included in multivariate analysis, after further reduction by stepwise backward method, variables corresponding to Akaike's Information Criteria (AIC) minimum value were included to form modeling variables.[25] When the Least Absolute Shrinkage and Selection Operator (LASSO) regression was used to screen variables, cross-validation was used to find the minimum criteria and the 1-se criteria for tuning parameter (λ), and independent variables corresponding to the 1-se criteria for λ were selected to get modeling variables, the final modeling variable was obtained by comprehensive analysis of the above results.[26] Draw forest plots and nomogram through modeling variables. Discrimination of the nomogram was assessed by the area under the receiver operating characteristic curve (ROC curve).[27] Calibration curve to evaluate the calibration of the predicted probability and actual probability. Decision curve to analyze the clinical application value of the model.[28] Kaplan-Meier (KM) curve to describe the difference between high, mediate and low risk of the model. In order to facilitate clinical use, the online

web version of the nomogram was established. Finally, evaluation the value of $CD8^+$T cell counts by analysing the effect of different treatments on the survival rate of $CD8^+$T subgroup and the changes of modeling indexes in 1-year follow-up.

Results

Demographic Characteristics

Table 1 showed the demographic and clinical characteristics of the baseline training cohort (n = 210) and validation cohort (n = 90). The KM curve demonstrated that there was no statistical difference in OS between the training set and the validation set after random grouping (p = 0.94) (Figure 1). Of the total 300 patients, 240 (80%) males; 61 (20.3%) patients were younger than 50 years old. In terms of personal and past medical history, 68.7% of the patients smoked, 65.3% drank alcohol. 24% had hypertension, 4% had coronary heart disease, and 28% had diabetes. For complications, 9.7% of patients had UGIB, 3.3% had hepatic encephalopathy, and 60.3% had portal hypertension. The majority of patients were treated with TACE (49.3%), and the majority of patients were caused by hepatitis B infection (85.3%), and more than half of the patients had normal liver function. In terms of T lymphocytes, T cell counts < 1027 cells/μL in 76.7% of patients, $CD8^+$T cell counts ≤ 320 cells/μL in 67.3% of patients, and $CD4^+$T cell counts ≤ 706 cells/μL in 83% of patients. On the whole, there was no statistically significant difference in each index between the two groups at baseline, indicating comparability (P > 0.05).

Model Specifications and Predictors of OS

Backward Stepwise Cox Regression to Select Optimal Prognostic Variables

In the training cohort, univariate Cox regression was used for univariate screening results, reasons, lymphocyte counts, NLR, ALT, AST, PTA, TBIL, INR, AFP, CRP, T cell counts, $CD4^+$T cell counts, and $CD8^+$T cell counts were significantly correlated with the survival of the training cohort (P < 0.05), after multivariate analysis and stepwise backward method, the minimum AIC value was 869.14, the corresponding modeling variables were AFP, CRP, AST, and $CD8^+$T cell counts (Table 2).

LASSO Method to Select Optimal Prognostic Variables

In the training cohort, 28 candidate predictors were brought into lasso regression. When the lambda. 1se = 0.165, the log (λ) = −1.802, the variables were selected through cross-validation, including AFP, CRP, AST, and $CD8^+$T cell counts, respectively. The model constructed by them was considered to have the advantage of excellent performance and minimum number of modeling variables (Figure 2).

Based on the above results, the following four variables were included in the model. AFP (> 400 ng/mL vs ≤ 400ng/mL; HR: 2.946; 95% CIs: 1.877–4.624; p < 0.001), CRP (≥ 5mg/L vs < 5mg/L; HR: 2.306; 95% CIs: 1.513–3.514; p < 0.001) and AST (> 40U/L vs ≤ 40U/L; HR: 2.21; 95% CIs: 1.443–3.384; p < 0.001) were risk factors affecting the prognosis of HCC, while $CD8^+$T cell counts (> 320 cells/μL vs ≤ 320 cells/μL; HR: 0.484; 95% CIs: 0.271–0.862; p = 0.0138) was protective factor (Figure 3A).

Construction of the Nomogram for 1-, 2-, and 3-Year OS

In nomogram for 1-, 2-, and 3-year OS, the four independent prognostic factors (AFP, AST, CRP, and $CD8^+$T cell counts) screened above were included (Figure 3B). Different horizontal axes represent different independent prognostic factors, the importance of the indicators is defined by standard deviation, and the importance increases from top to bottom. According to the actual patient's indicators, the corresponding index scores were found and summed up, drawing the total score of axis vertical line, the point where the vertical line intersects the survival axis is the predicted survival rate, and the higher the score, the worse the prognosis, the nomogram also shows the confidence interval range.

Nomogram Validation and Assessment

ROC curve was drawn to evaluate the discrimination of the model. The area under the curve (AUC) of 1-, 2-, and 3-year OS was 0.846 (95% CIs: 0.785–907), 0.824 (95% CIs: 0.766–0.882) and 0.812 (95% CIs: 0.756–0.869) respectively in the training cohort (Figure 4A) and 0.877 (95% CIs: 0.793–0.961), 0.794 (95% CIs: 0.690–0.899) and 0.789 (95% CIs: 0.688–

Table 1 Characteristics of Patients with Small Hepatocellular Carcinoma

Demographic and Clinical Values	Total n=300(%)	Training Cohort n=210(%)	Validation Cohort n=90(%)	P-value
Sex				0.208
Male	240(80.0)	172(81.9)	68(75.6)	
Female	60(20.0)	38(18.1)	22(24.4)	
Age (year)				0.827
≤50	61(20.3)	42(20.0)	19(21.1)	
>50	239(79.7)	168(80.0)	71(78.9)	
Smoking				0.303
Yes	206(68.7)	148(70.5)	58(64.4)	
No	94(31.3)	62(29.5)	32(35.6)	
Drinking				0.459
Yes	196(65.3)	140(66.7)	56(62.2)	
No	104(34.7)	70(33.3)	34(37.8)	
Hypertension				0.860
Yes	72(24.0)	51(24.3)	21(23.3)	
No	228(76.0)	159(75.7)	69(76.7)	
Heart disease				0.095
Yes	12(4.0)	11(5.2)	1(1.1)	
No	288(96.0)	199(94.8)	89(98.9)	
Diabetes				0.145
Yes	84(28.0)	64(30.5)	20(22.2)	
No	216(72.0)	146(69.5)	70(77.8)	
UGIB				0.469
Yes	29(9.7)	22(10.5)	7(7.8)	
No	271(90.3)	188(89.5)	83(92.2)	
Hepatic encephalopathy				1.000
Yes	10(3.3)	7(3.3)	3(3.3)	
No	290(96.7)	203(96.7)	87(96.7)	
Portal hypertension				0.396
Yes	181(60.3)	130(61.9)	51(56.7)	
No	119(39.7)	80(38.1)	39(43.3)	
Treatments				0.953
TACE	148(49.3)	104(49.5)	44(48.9)	
RFA	75(25.0)	52(24.8)	23(25.6)	
TACE+RFA	77(25.7)	54(25.7)	23(25.6)	
Reasons				0.305
HBV	256(85.3)	182(86.7)	74(82.2)	
HCV	17(5.7)	11(5.2)	6(6.7)	
Alcohol	18(6.0)	12(5.7)	6(6.7)	
Other	9(3.0)	5(2.4)	4(4.4)	
Child-Pugh Stage				0.914
A	204(68.0)	142(67.6)	62(68.9)	
B	90(30.0)	65(31.0)	25(27.8)	
C	6(2.0)	3(1.4)	3(3.3)	
WBC (10^9/L)				0.920
≤4	142(47.3)	99(47.1)	43(47.8)	
>4	158(52.7)	111(52.9)	47(52.2)	
Neutrophil count (10^9/L)				0.801
≤2.37	150(50.0)	104(49.5)	46(51.1)	
>2.37	150(50.0)	106(50.5)	44(48.9)	

(*Continued*)

Table 1 (Continued).

Demographic and Clinical Values	Total n=300(%)	Training Cohort n=210(%)	Validation Cohort n=90(%)	P-value
Lymphocyte count (10^9/L)				0.687
≤0.98	152(50.7)	108(51.4)	44(48.9)	
>0.98	148(49.3)	102(48.6)	46(51.1)	
NLR				0.669
≤2.5	151(50.3)	104(49.5)	47(52.2)	
>2.5	149(49.7)	106(50.5)	43(47.8)	
ALP (U/L)				0.606
≤45	9(3.0)	7(3.3)	2(2.2)	
>45	291(97.0)	203(96.7)	88(97.8)	
PLT (10^9/L)				0.332
≤100	176(58.7)	127(60.5)	49(54.4)	
>100	124(41.3)	83(39.5)	41(45.6)	
ALT (U/L)				0.734
≤50	237(79.0)	167(79.5)	70(77.8)	
>50	63(21.0)	43(20.5)	20(22.2)	
AST (U/L)				0.092
≤40	201(67.0)	147(70.0)	54(60.0)	
>40	99(33.0)	63(30.0)	36(40.0)	
TBIL (μmol/L)				0.529
≤18.8	145(48.3)	104(49.5)	41(45.6)	
>18.8	155(51.7)	106(50.5)	49(54.4)	
PTA (%)				0.866
≤70	82(27.3)	58(27.6)	24(26.7)	
>70	218(72.7)	152(72.4)	66(73.3)	
INR				0.269
≤1.2	181(60.3)	131(62.4)	50(55.6)	
>1.2	119(39.7)	79(37.6)	40(44.4)	
AFP (ng/mL)				0.394
≤400	220(73.3)	157(74.8)	63(70.0)	
>400	80(26.7)	53(25.2)	27(30.0)	
CRP (mg/L)				0.657
≤5	191(63.7)	132(62.9)	59(65.6)	
>5	109(36.3)	78(37.1)	31(34.4)	
T cell counts (cells/μL)				0.766
<1027	230(76.7)	162(77.1)	68(75.6)	
≥1027	70(23.3)	48(22.9)	22(24.4)	
$CD8^+$T cell counts (cells/μL)				0.148
≤320	202(67.3)	136(64.8)	66(73.3)	
>320	98(32.7)	74(35.2)	24(26.7)	
$CD4^+$T cell counts (cells/μL)				0.569
≤706	249(83.0)	176(83.8)	73(81.1)	
>706	51(17.0)	34(16.2)	17(18.9)	
CD4/CD8(%)				0.752
≤1.5	106(35.3)	73(34.8)	33(36.7)	
>1.5	194(64.7)	137(65.2)	57(63.3)	

Abbreviations: WBC, white blood cells; ALP, alkaline phosphatase; HBV, hepatitis B virus; HCV, hepatitis C virus; PLT, platelet counts; UGIB, upper gastrointestinal hemorrhage; NLR, neutrophil-lymphocytes ratio; ALT, alanine aminotransferase; TBIL, total bilirubin; PTA, prothrombin time activity; AFP, alpha-fetoprotein.

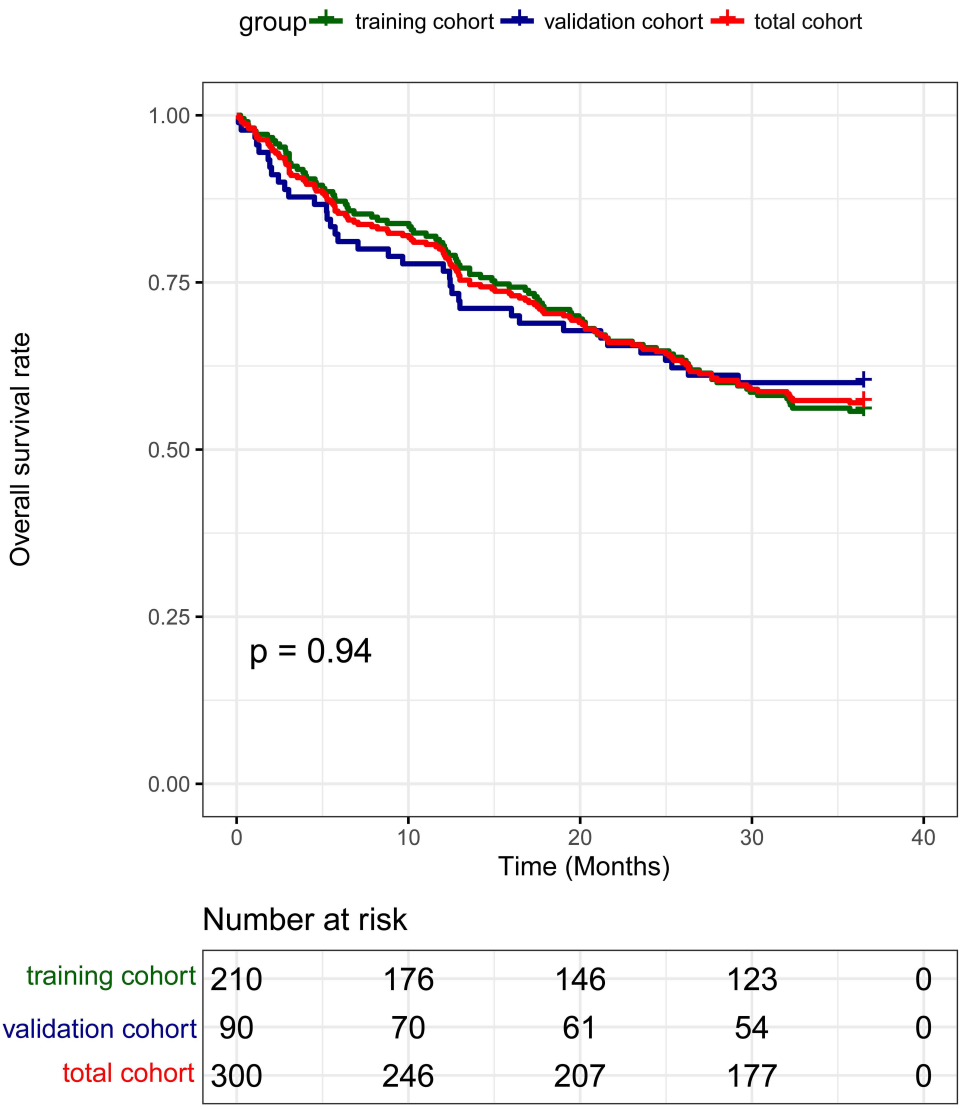

Figure 1 Kaplan–Meier survival curve of enrolled small HCC patients, training and validation cohort.

0.890) in the validation cohort (Figure 4D). Compared with other classical tumor prediction models (JIS, Okuda, TNM, CLIP and CUPI), the nomogram showed better discrimination in both training (Figure 4B) and validation (Figure 4E) set. Bootstrap validation with 1000 resampling was performed to assess model accuracy and potential model overfitting, the 1-year, 2-year and 3-year bootstrapped calibration plot indicate that there is a strong consistency between actual observation and prediction (Figure 3C and D). The clinical applicability of the model was evaluated by decision curve analysis (DCA), which showed a higher net benefit compared to the four independent predictors (Figure 4C and F).

Risk Stratification

To further verify the predictive power of the model, we stratified the risk of the population. The stratification method was based on the nomogram scores, patients were divided into low-risk (< 113 scores), medium-risk (113–196 scores), and high-risk (≥ 196 scores) groups. The KM curve indicated that the model could distinguish high, middle and low risk populations well in both training ($p < 0.0001$) and validation sets ($p < 0.0001$) (Figure 5A and B). To further explore the discrimination ability in $CD8^+$T subgroups, we divided $CD8^+$T index into high (> 320 cells/μL) and low (≤ 320 cells/μL) groups, the KM curve showed that, in the low $CD8^+$T cell counts subgroup, the model can effectively divide the high, medium and low risk groups both in the training set ($p < 0.0001$) and the validation set ($p < 0.0001$) (Figure 5C and D),

Table 2 Univariate and multivariate Cox Regression Analyses for 3-Year OS in Patients with Small Hepatocellular Carcinoma

Characteristics	Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	P values	HR (95% CI)	P values
Sex, male vs.female	0.81 (0.47–1.41)	0.451		
Age, ≤50 vs.>50	1.04 (0.62–1.75)	0.868		
Smoking, no vs.yes	1.01 (0.65–1.57)	0.978		
Drinking, no vs.yes	0.88 (0.57–1.34)	0.541		
UGIB, no vs.yes	1.35 (0.72–2.54)	0.344		
Hypertension, no vs.yes	0.99 (0.61–1.61)	0.959		
Diabetes, no vs.yes	1.13 (0.73–1.75)	0.582		
Heart disease, no vs.yes	0.87 (0.32–2.38)	0.793		
Hepatic encephalopathy, no vs.yes	1.4 (0.51–3.82)	0.509		
Treatment				
TACE	Ref			
RFA	0.92 (0.56–1.51)	0.754		
TACE+RFA	0.7 (0.42–1.18)	0.182		
Reasons				
HBV	Ref			
HCV	2.17 (1.04–4.5)	0.038		
Alcohol	0.75 (0.27–2.05)	0.576		
Other	1.94 (0.61–6.14)	0.262		
WBC (10^9/L), ≤4 vs.>4	0.88 (0.58–1.32)	0.531		
Neutrophil counts (10^9/L), ≤2.37 vs.>2.37	1.01 (0.67–1.51)	0.974		
Lymphocyte counts (10^9/L), ≤0.98 vs.>0.98	0.48 (0.31–0.73)	0.001		
NLR, ≤2.5 vs.>2.5	1.77 (1.17–2.68)	0.007		
PLT (10^9/L), ≤100 vs.>100	0.85 (0.56–1.3)	0.461		
ALT (U/L), ≤50 vs.>50	2.75 (1.78–4.25)	< 0.001		
AST (U/L), ≤40 vs.>40	3.29 (2.19–4.96)	< 0.001	2.21 (1.44–3.38)	<0.001
PTA (%), ≤70 vs.>70	0.62 (0.41–0.95)	0.03		
TBIL (μmol/L), ≤18.8 vs.>18.8	1.97 (1.3–3)	0.002		
ALP (U/L), ≤45 vs.>45	1.83 (0.45–7.44)	0.397		
INR, ≤1.2 vs.>1.2	1.53 (1.01–2.3)	0.043		
AFP (ng/mL), ≤400 vs.>400	4.18 (2.76–6.32)	< 0.001	2.94 (1.88–4.62)	<0.001
CRP (mg/L), ≤5 vs.>5	2.81 (1.87–4.23)	< 0.001	2.31 (1.51–3.51)	<0.001
T cell counts (cells/μL), <1027 vs.≥1027	0.5 (0.28–0.88)	0.017		
$CD4^+$T cell counts (cells/μL), ≤706 vs.>706	0.48 (0.24–0.95)	0.035		
$CD8^+$T cell counts (cells/μL), ≤320 vs.>320	0.29 (0.17–0.5)	< 0.001	0.48 (0.27–0.86)	0.0138
CD4/CD8 (%), ≤1.5 vs.>1.5	1.24 (0.8–1.93)	0.34		

Abbreviations: WBC, white blood cells; ALP, alkaline phosphatase; HBV, hepatitis B virus; HCV, hepatitis C virus; PLT, platelet counts; UGIB, upper gastrointestinal hemorrhage; NLR, neutrophil-lymphocytes ratio; ALT, alanine aminotransferase; TBIL, total bilirubin; PTA, prothrombin time activity; AFP, alpha-fetoprotein; HR, hazard ratio; TACE, trans-arterial chemoembolization; RFA, radiofrequency ablation.

and in the high $CD8^+$T cell counts subgroup, the model could only distinguish the training set significantly (p = 7e-04) (Figure 5E) and lack of high-risk groups in the validation set (p = 0.26) (Figure 5F).

Online Web Version of the Nomogram

In order to improve the clinical value of the model, the web version of the nomogram (Figure 6) was established, which could be accessed through the following website (https://hccnomogran.shinyapps.io/DynNomapp/), clinicians can obtain the predicted survival probability of patients at different time points by inputting the corresponding index values of patients.

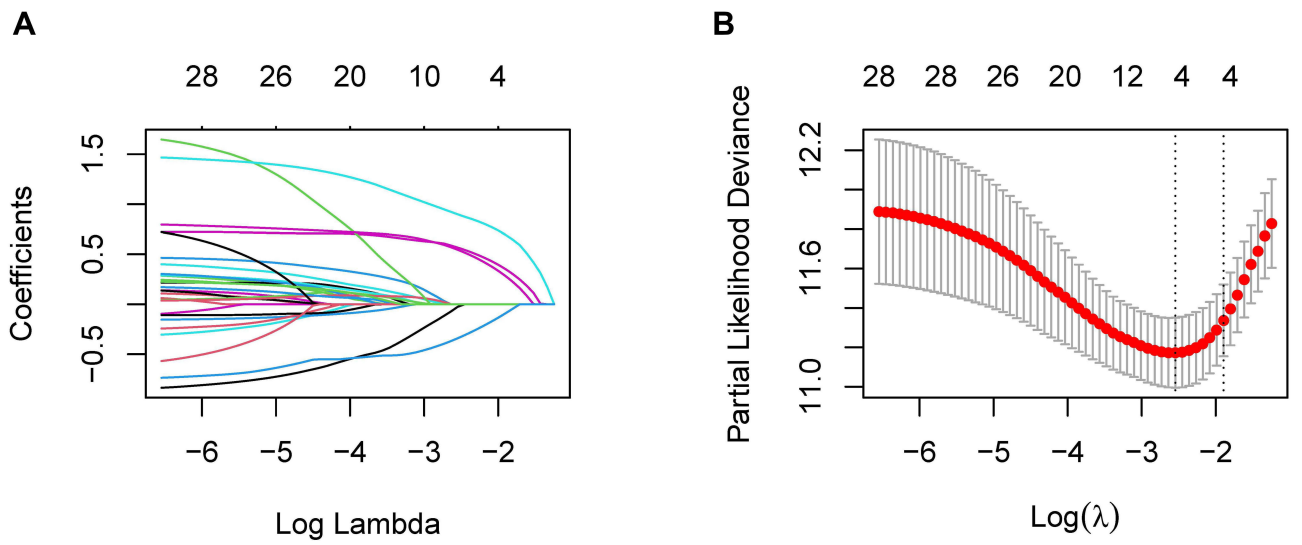

Figure 2 LASSO Method to select Optimal Prognostic Variables.
Notes: (**A**) LASSO model coefficient trendlines of the 28 variables for OS. (**B**) λ selection cross-validation error curve. Vertical lines were drawn at the optimal values given by the lambda (λ). min and lambda (λ).1se. When lambda.1se = 0.165, the log (λ) = −1.802, the variables were selected, including four indexes.
Abbreviations: LASSO, least absolute shrinkage and selection operator; OS, overall survival; se, standard error.

Evaluation of $CD8^+$T Cell Counts Value

The effect of different treatments on the survival rate of $CD8^+$T subgroup was further analyzed. In the total cohort, the survival rate of patients receiving RFA ($p = 0.0043$) and RFA+TACE ($p = 0.0005$) was significantly different. In the training cohort, there was also a difference in RFA ($p = 0.00046$) and TACE+RFA ($p < 0.0001$) group. In the validation cohort, no difference in survival was observed under any of the three treatments (Figure S1). After 1 year of follow-up, $CD8^+$T cell counts levels were not significantly different from baseline, nor were other modeling indexes (Figure S2).

Discussion

In this study, a survival model of small HCC after minimally invasive treatment was established. The construction of this model combined two modeling methods. In backward stepwise Cox regression, modeling variables were screened by univariate analysis, multivariate analysis and stepwise backward method based on AIC just according to the existing training set data, variables with no significant correlation with the results were gradually eliminated and modeling variables were finally obtained, this may lead to the phenomenon of over-fitting of the model. Unlike Cox regression, LASSO was more suitable for survival data analysis with high dimension, strong correlation and small sample size. By compressing some meaningless or insignificant variable coefficients to 0, a better model could be obtained, then through cross validation to fully consider factors beyond the data set, which could effectively avoid the model fitting problem.[26] Based on the above methods, we obtained four independent influencing factors (AST, AFP, CRP, and $CD8^+$T cell counts), representing liver function index, tumor-related index, inflammation index, and immune-related index, respectively. Thus, the model took into account various factors that may affect the prognosis of patients with small HCC after minimally invasive treatment, demonstrating its excellent comprehensive discrimination ability.

The prognosis of HCC patients is influenced by many clinical factors, including liver function, tumor biological characteristics and patient immune status. It is an undoubted conclusion that liver function can affect the prognosis of patients with HCC and better liver function can often bring about longer OS. A number of HCC prognostic models have been established based on liver function indicators, including: APRI (AST/PLT) and ALRI (AST/lymphocyte) have a good prediction effect in predicting liver failure after hepatectomy. With the increase of the score, the prognosis of patients is worse, which is consistent with the results of our study.[29] Different from our model, their formation is only based on the ratio of the two indicators, so the scope of consideration is not comprehensive enough, leading to their limited clinical applicability.

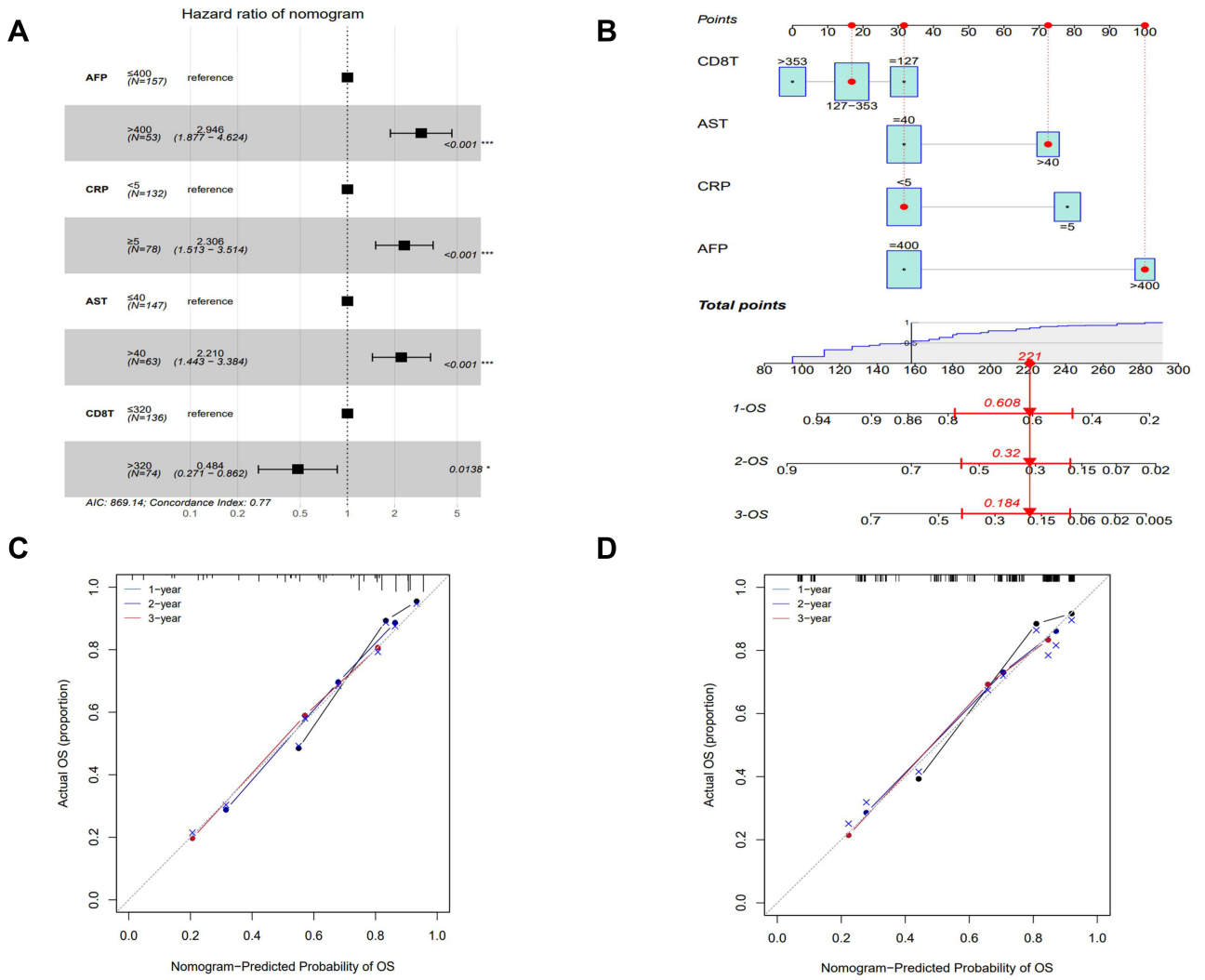

Figure 3 Forest plot with hazard ratio for the modeling variables, Nomogram in the training cohort and Calibration curve.
Notes: (**A**) In the Forest plot, HRs above one indicates that a variable is negatively with survival time, and *indicates the significance of the P value (***: P < 0.001; *: P < 0.05); (**B**) In the nomogram, different horizontal axes represent different independent prognostic factors. According to the actual patient's indicators, the corresponding index scores were found and summed up, drawing the total score of axis vertical line, the point where the vertical line intersects the survival axis is the predicted survival rate; The calibration curves for 1-,2- and 3-year OS were identified in the training cohort (**C**) and in the validation cohort (**D**), shown the model has good calibration ability between the predicted and actual observation.
Abbreviations: AIC, Akaike's Information Criteria; OS, overall survival; CD8T, $CD8^+$T cell counts.

AFP is secreted by newborn naive hepatocytes and produced in large quantities when hepatocytes become cancerous, which is often used as an oncology indicator in the diagnosis of HCC and is widely used.[7,8] But in the clinical, 30–40% of HCC patients show negative AFP characteristics, such patients tend to have low AFP expression due to factors such as high degree of tumor differentiation, early TNM stage, small tumor size. More importantly, such patients often account for a large proportion of people with small HCC.[30] Therefore, AFP alone has limitations in the diagnosis and prognosis of small HCC, needs to be combined with other factors to distinguish.

Chronic inflammation is the basis of HCC, oxidative damage and the increase of inflammatory mediators can promote the development of inflammation, leading to cancer.[31] Previous reports have confirmed that liver injury is caused by oxidative stress of hepatocytes.[32,33] Therefore, antioxidants may have a certain application prospect in the clinical treatment of liver diseases in the future. At the same time, some studies have shown that RFA may aggravate inflammation and oxidative stress in the early stage of HCC treatment. When combined with TACE, RFA can improve the inflammatory state of the body and significantly improve the antioxidant capacity of the body.[34] Therefore, the effect of combination therapy is more recommended than treatment alone, and this view is supported by several meta-analyses.[35] This is also consistent with the

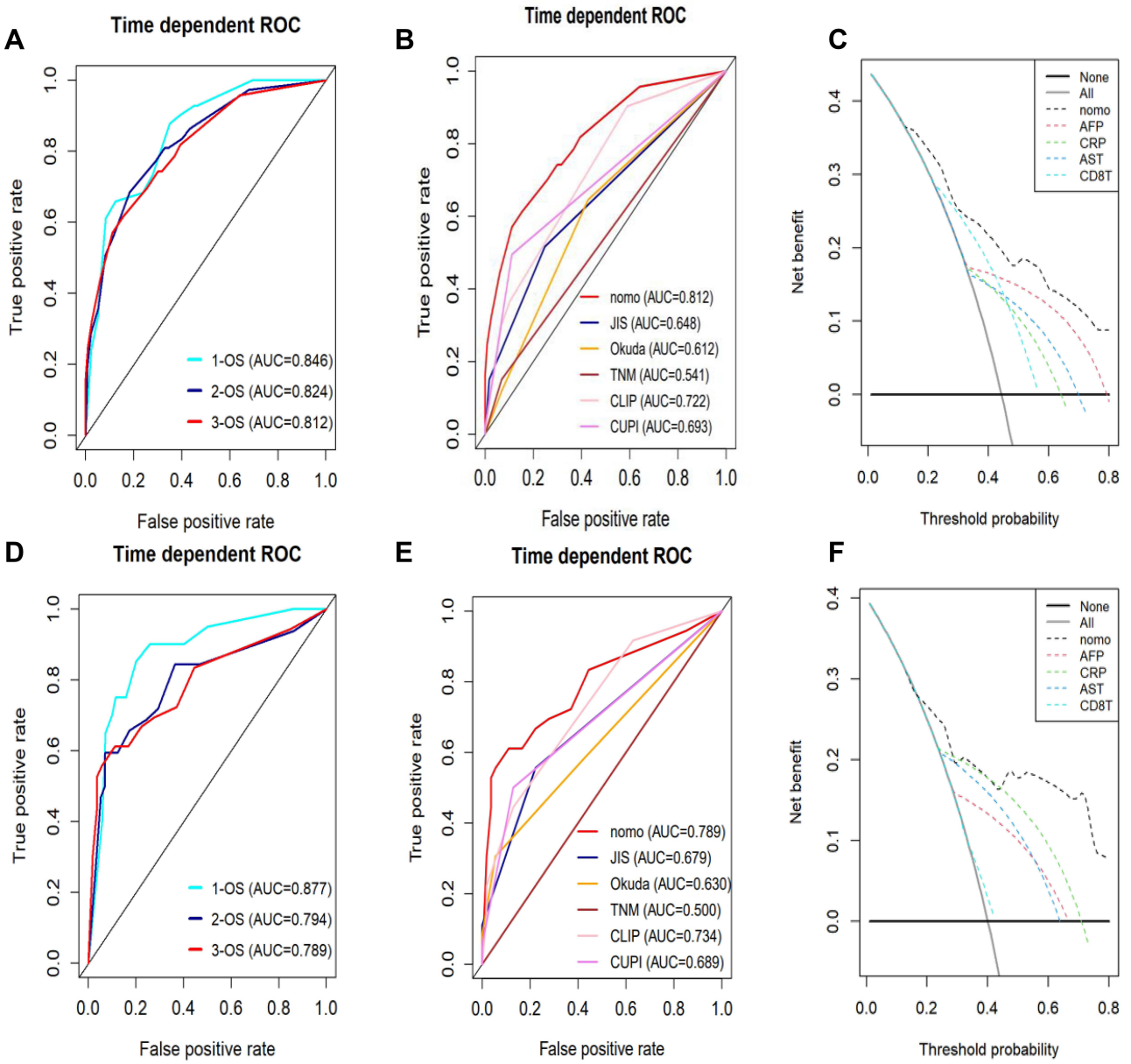

Figure 4 ROC curve and and DCA of the nomogram.

Notes: (**A**) ROC curves of 1-, 2-, and 3-year OS for assessing the discrimination ability of nomogram in the training cohort; (**B**) ROC curves for assessing the discrimination ability of nomogram from other classical models in the training cohort; (**C**) DCA showed that the nomogram had wider threshold probabilities and yielded more net benefit than the four independent predictors in the training cohort; (**D**) ROC curves of 1-, 2-, and 3-year OS for assessing the discrimination ability of nomogram in the validation cohort; (**E**) ROC curve for assessing the discrimination ability of nomogram from other classical models in the validation cohort; (**F**) DCA showed that the nomogram had wider threshold probabilities and yielded more net benefit than the four independent predictors in the validation cohort.

Abbreviations: OS, overall survival; AUC, the area under the curve; DCA, decision curve analysis; nomo, nomogram; CUPI, the Chinese University Prognostic Index; JIS, Japan Integrated Staging; TNM, tumor–lymph node–metastasis; CLIP, Cancer of the Liver Italian Program; AST, aspartate aminotransferase; AFP, alpha fetoprotein; CRP, C-reactive protein.

results of subgroup analysis in our study. CRP is a biomarker of inflammation,[36] which can reflect the deterioration of the disease and the degree of inflammation. For cancer patients, the level of CRP can reflect the severity of the cancer, and the higher the CRP level, the more malignant the cancer will be. It has been successfully used as a prognostic factor for several malignant tumors.[37,38] In the field of HCC, studies have also shown that AFP combined with CRP can be used to predict the prognosis of HCC.[39] More importantly, Overexpressed CRP has more significant prognostic significance for patients with AFP negative.[40] In this study, CRP was also found to be an independent prognostic factor for small HCC (HR=2.306, 95% CI: 1.513–3.514). The higher the CRP, the worse the prognosis of patients. In the whole model, CRP accounted for a large

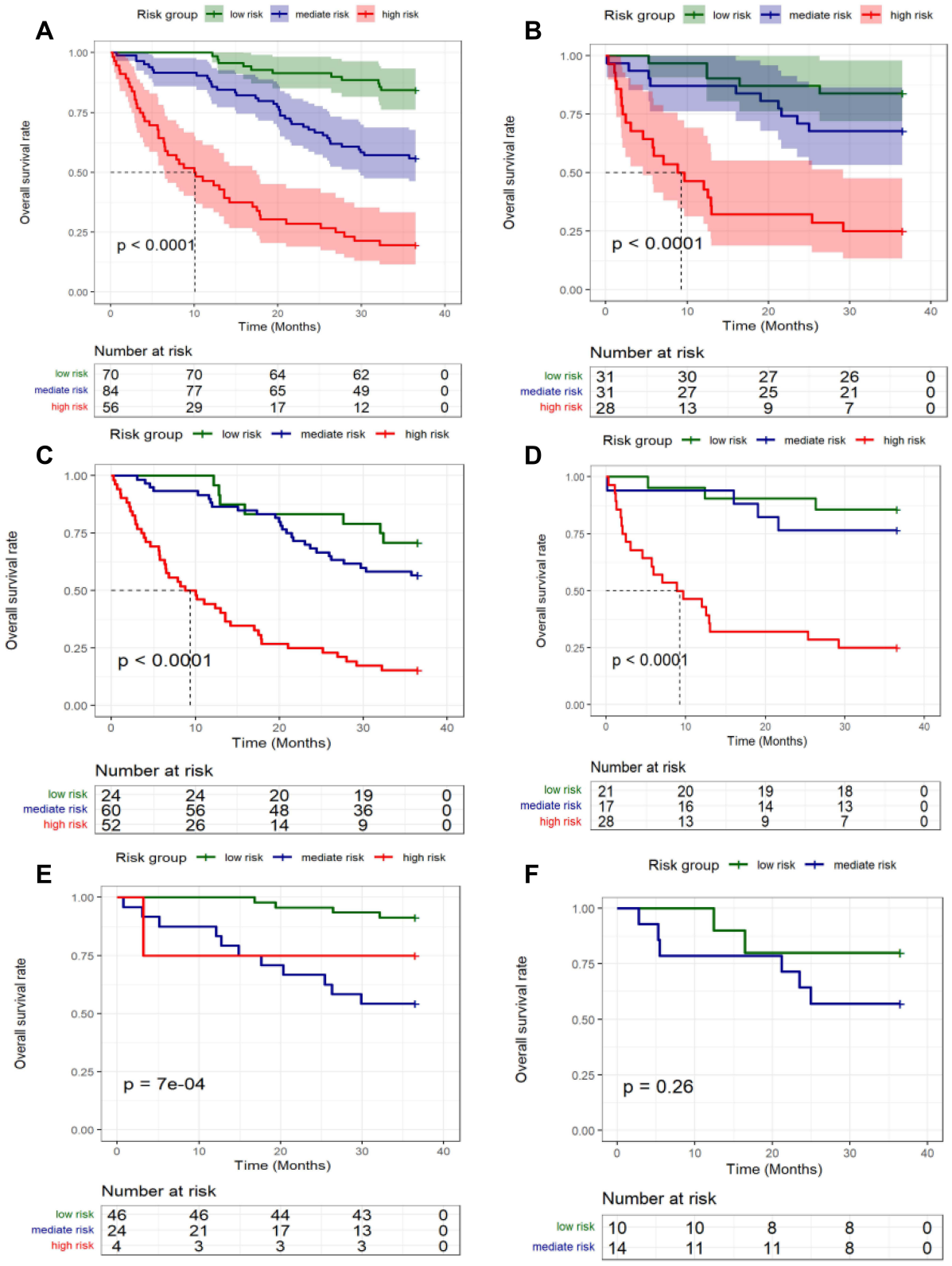

Figure 5 Risk stratification.

Notes: The nomogram score is divided into low-, medium-, and high-risk groups in the training cohort (**A**) and validation cohort (**B**); When $CD8^+$T cell counts ≤ 320 cells/μL, the condition of Risk stratification in the training cohort (**C**) and validation cohort (**D**); When $CD8^+$T cell counts > 320 cells/μL, the condition of Risk stratification in the training cohort (**E**) and validation cohort (**F**).

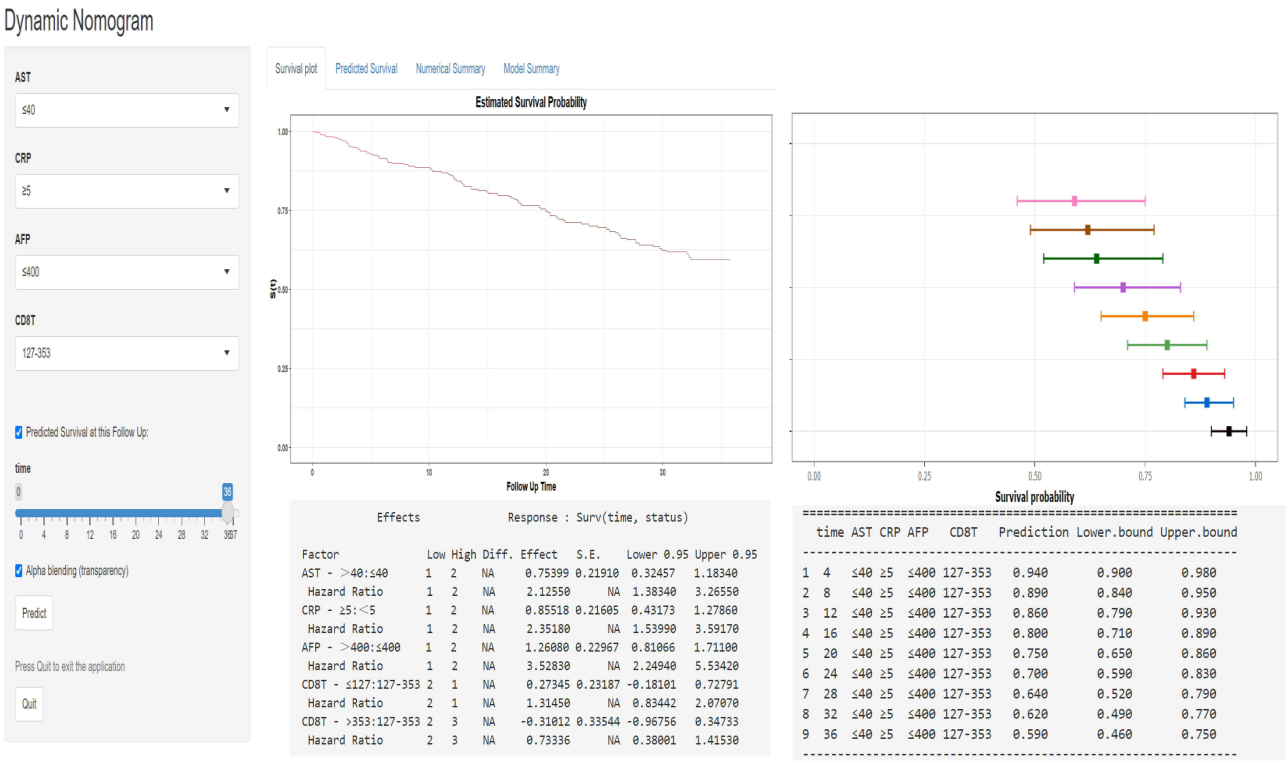

Figure 6 Online web version of the nomogram.
Notes: A table predicting the probability of survival and its confidence interval range can be obtained by entering the actual patient index values.

proportion and had a great influence. Compared with the model only used to predict the prognosis of AFP negative HCC, this model is more generally suitable for small HCC patients.

In recent years, with the in-depth understanding of tumor immune microenvironment, the role of immune regulation in the monitoring and control of malignant tumors has been fully affirmed.[41] Tumor-infiltrating lymphocytes (TILs) play an important role in the immune microenvironment[42] and are significantly correlated with prognosis.[43] Their anti-tumor and tumor-promoting abilities are related to a variety of T lymphocytes in the immune microenvironment, among which $CD8^+$T cells are the most common T lymphocytes.[44] It can directly kill tumor cells by through synaptic exocytosis of cytotoxic granules containing perforin and granzymes. It can also kill tumor cells indirectly by secreting cytokines (such as: Interferon-γ (IFN-γ) and Tumor Necrosis Factor alpha (TNF-α).[45] It was found that the $CD8^+$T cell exhaustion in cancer inhibited the anti-tumor immune function of patients and affected the therapeutic effect of patients.[46] Previous studies[47] have shown that there are fewer $CD8^+$T cells in intratumoral compared with periatumoral, and a high level of $CD8^+$T cells is considered to be a good prognostic indicator of hepatocellular carcinoma.[48] In this study, lymphocyte counts and T lymphocytes (T cell counts, $CD4^+$T cell counts, $CD8^+$T cell counts and CD4/CD8) were all included in the candidate single factors. Only $CD8^+$T cell counts (HR=0.484, 95% CI: 0.271–0.862) was included in the final model. The higher the level of $CD8^+$T cells, the better the prognosis of patients, the high level of $CD8^+$T cell counts was a protective factor for patients with HCC. Our results are consistent with those described in the literature. Although there are many immune-related prognostic models in the field of HCC, most of them are established based on immune-related gene difference analysis.[49] However, gene detection technology is not widely used in clinical practice and is mostly restricted by region, which is mostly seen in developed regions, so a routine detection system has not yet been formed. Compared with them, detection of T lymphocytes is simpler and faster, and has become a routine test in most hospitals.

The $CD8^+$T subgroup was investigated in this study. In risk stratification, the risk discrimination ability of the model was not reflected in the validation cohort of the high level $CD8^+$T group. The reason may be that the total population of the validation set in this study was too small, and the disease of small HCC patients was relatively mild, so that the number of high-risk populations was 0, which failed to demonstrate good discrimination ability. When comparing the survival rates of $CD8^+$T subgroups among

different treatments, only the RFA and RFA+TACE groups in the total cohort and training cohort showed significant differences in survival rates, which may be reflected that compared with those receiving TACE treatment, this model is more significant for RFA and combination therapy in predicting survival in patients with small HCC. Also, due to the limitation of the number of patients, this phenomenon was not reflected in the validation cohort, and large-scale subsequent trials are needed to verify the results of this study.

Minimally invasive treatment is the main treatment for small HCC, but it also has some shortcomings. RFA often causes tumor recurrence due to incomplete local ablation, while TACE may form local hypoxia while forming arterial embolism, which can promote the generation of peripheral blood vessels and contribute to the development of tumor. The emergence of new technology is very necessary. With the development of precision medicine, various nanotechnology has been widely explored to minimize the toxic and side effects of treatment by precisely targeting tumor areas. It has been reported that ablative immunotherapy based on mesoporous silica nanosystems can effectively improve anti-tumor immunity of patients by inducing reactive oxygen species to enhance immunogenic cell death.[50] This model combines the immune index ($CD8^+$T cell counts) with other clinical indicators to pay more attention to the immune function of patients. Meanwhile, a web version of the prediction model (https://hccnomogran.shinyapps.io/DynNomapp/) is also made, which is more convenient for clinical promotion and use. It is believed that both the existing minimally invasive technology and the use of new technologies in the future have good predictive power.

The model established in this study has good performance in discrimination, calibration and clinical applicability in both the training cohort and the validation cohort, and comprehensively considers three aspects that affect the prognosis of hepatocellular carcinoma. It includes liver function, tumor biological characteristics and patient immune status. Compared with other models, the prediction effect of T lymphocytes is emphasized, which can assist in the prediction of small HCC after minimally invasive treatment. At the same time, we must also admit that this model has the following limitations: ① This study is a single-center, small-sample retrospective study, lacking external validation, requiring multi-center, prospective external trials to verify our results. ② The population of this study was all Chinese patients, and it is not clear whether it can be applied to patients in other countries. ③ This study only has 1-year follow-up data of modeling indicators, which fails to reflect the long-term changes of indicators in detail. Data can be collected for further analysis.

Conclusion

Based on $CD8^+$T cell counts, a prognostic model for small HCC after minimally invasive treatment was established. Compared with classical models such as JIS, Okuda, TNM, CLIP and CUPI, it was found that $CD8^+$T cell counts in T lymphocytes may be a reliable indicator for predicting the survival of small HCC patients. Subsequent larger trials are needed to verify the results.

Abbreviations

HCC, small hepatocellular carcinoma; TACE, Trans-arterial chemoembolization; AASLD, the American Association for the Study of Liver Diseases; EASL, the European Association for the Study of the Liver; AIC, Akaike's Information Criteria; LASSO, the Least Absolute Shrinkage and Selection Operator; DCA, decision curve analysis; UGIB, upper gastrointestinal hemorrhage; AST, aspartate aminotransferase; AFP, alpha fetoprotein; CRP, C-reactive protein; ROC curve, Receiver operating characteristic curve; AUC, The area under the curve; RFA, radiofrequency ablation; Treg cells, regulatory T cells; TNF-α, Tumor Necrosis Factor alpha; IFN-γ, Interferon-γ; WBC, white blood cells; NLR, neutrophil-lymphocytes ratio; ALP, alkaline phosphatase; PLT, platelet counts; INR, International Normalized Ratio; ALT, alanine aminotransferase; TBIL, total bilirubin; PTA, prothrombin time activity; KM curve, Kaplan-Meier curve; HR, hazard ratio; OS, overall survival; CUPI, the Chinese University Prognostic Index; JIS, Japan Integrated Staging; TNM, the tumor node metastasis staging; CLIP, Cancer of the Liver Italian Program.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Beijing Ditan Hospital affiliated to Capital Medical University. All procedures involving human experiments were carried out in accordance with the Declaration of Helsinki, and every patient participating in the study signed an informed consent.

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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