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An elevated percentage of CD4$^+$CD25$^+$CD127^{low} regulatory T cells in peripheral blood indicates a poorer prognosis in hepatocellular carcinoma after curative hepatectomy

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Abstract

Background Previous studies suggest the percentage of CD4$^+$CD25$^+$CD127^{low} regulatory T cells (Tregs) in peripheral blood of patients with hepatocellular carcinoma (HCC) was significantly higher than that in healthy, which may be a significant predictor of HCC clinical outcome, and we examined the utility of Tregs in predicting prognosis in HCC after curative hepatectomy.

Methods 77 diagnosed HCC patients from August 2018 to March 2023 were selected as research objects, we retrospectively analyzed whether the preoperative percentage of CD4$^+$CD25$^+$CD127low Tregs in peripheral blood predicts prognosis after curative hepatectomy in HCC patients. The percentage of CD4$^+$CD25$^+$CD127^{low} Tregs was detected by flow cytometry.

Results The percentage of CD4$^+$CD25$^+$CD127^{low} Tregs was significantly elevated in patients who developed recurrence and death ($p < 0.050$). X-tile software was used to calculate optimal cut-off value of Treg percentage (5.85%), and patients were divided into two groups with high and low Treg percentage. Patients with higher preoperative Treg percentage had a significantly poorer prognosis ($p < 0.050$). Cox regression demonstrated the percentage of CD4$^+$CD25$^+$CD127^{low} Tregs was an independent indicator for poor prognosis after hepatectomy. The Recurrence-free survival (RFS) (the log-rank test, $p < 0.001$) and Overall survival (OS) (the log-rank test, $p = 0.008$) in patients with higher Treg percentage were significantly lower than that in patients with lower Treg percentage. The results were confirmed by the subgroup analysis.

Conclusion The percentage of CD4$^+$CD25$^+$ CD127^{low} Tregs in peripheral blood is associated with poor prognosis in HCC patients. It can be suggested as a potential prognostic indicator for HCC patients after hepatectomy and complement existing risk stratification tools. Measuring the percentage of CD4$^+$CD25$^+$ CD127^{low} Tregs may contribute to the formulation of treatment strategies and the improvement of the prognosis for HCC patients.

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Keywords $CD4^+CD25^+CD127^{low}$ regulatory T cells, Hepatocellular carcinoma, Hepatectomy, Early recurrence, Prognosis

Introduction

Liver cancer is the fifth most common cancer and the second most frequent cause of cancer-related death globally. Hepatocellular carcinoma (HCC) represents about 90% of primary liver cancers and constitutes a major global health problem [1]. China has a high incidence of HCC, accounting for more than 50% of the world's incidence. According to the latest statistics, HCC ranks the second among tumor-related deaths in China, second only to lung cancer [2]. Hepatectomy is still the most commonly used potential radical treatment for HCC, but the recurrence rate of 5 years after surgery has been reported to be >70% [3]. In consequence, effective and timely prevention of recurrence is of great significance to reduce mortality and improve overall survival (OS).

Regulatory T cells (Tregs), which belong to a subgroup of CD4 + T cells, can maintain the homeostasis of the immune system by inhibiting the overactivation of other immune cells [4]. In tumor patients, Tregs may have a "double-edged sword effect", that is, the dual effect of inhibiting tumor cells and inhibiting anti-tumor immunity [5, 6]. Studies have shown that Tregs are enriched in peripheral blood, tumor tissue and adjacent tissues of HCC patients, and their percentage is significantly higher than that of healthy people. $CD4^+CD25^+CD127^{low}$ Tregs are the most specific Tregs populations in human peripheral blood, which has been confirmed to be significantly increased in HCC patients [7–8]. Although previous studies [9, 10] have confirmed that Tregs in the tumor microenvironment is negatively correlated with prognosis of HCC patients [11]. However, Tregs from different locations contribute to different clinical outcomes in patients [12]. For example, in colorectal cancer, Tregs localization (peripheral blood, tumor tissue and adjacent tissues) impacts patient clinical outcome differently [13]. Furthermore, Treg percentage in tumor microenvironment of HCC patients is difficult to detect before surgery. Therefore, its guiding significance for clinical treatments of HCC patients is limited, and it is more used to study immune mechanism of tumors [14, 15]. At present, there is a limited amount of research available on the correlation between Treg percentage in peripheral blood of HCC patients for their postoperative prognosis. Therefore, in this study, flow cytometry was used to detect Treg percentage in peripheral blood of HCC patients at the time of initial diagnosis, aiming to explore the clinical significance of Tregs in the prognosis of HCC patients after surgery.

Materials and methods

Patients' inclusion criteria and characteristics

We retrospectively selected 164 patients diagnosed with HCC in our hospital from August 2018 to May 2023, and we routinely tested for Treg percentage in peripheral blood. Inclusion criteria: (1) Patients who underwent curative anatomic hepatectomy performed by the same surgical team. (2) Primary HCC was confirmed by pathological examination after operation. (3) There were no tumor related treatments before surgery; Exclusion criteria: (1) Combined with other tumors or secondary tumors; (2) Positive margins. (3) Clinicopathological data were incomplete or lost to follow-up. (4) Perioperative death. After evaluation and consideration of families wishes, 108 patients received radical hepatectomy, 21 patients were excluded for preoperative tumor-related treatment, and postoperative pathology indicated that there were 7 cases with other tumors or secondary tumors, 2 cases of perioperative death, and 1 case of loss of follow-up. Finally, 77 patients (63 males and 14 females) were enrolled in the study, all were undergoing radical anatomic hepatectomy for the first time. Clinicopathologic characteristics of enrolled patients included: Basic information: sex, age; Preoperative laboratory indicators: prothrombin time (PT), albumin (ALB), total bilirubin (TBIL), alpha-fetoprotein (AFP), HBV-DNA; Operation: hepatectomy, operation time, blood loss; Postoperative pathological indicators: tumor diameter, histologic grade of differentiation, satellite nodules, microvascular invasion (MVI); Prognosis: recurrence-free survival (RFS), overall survival; Recurrent patients received antitumor therapy: Re-hepatectomy (repeated hepatectomy), transcatheter arterial chemoembolization (TACE), targeted therapy, and immunotherapy.

Patients follow-up

OS was calculated from the date of surgical intervention to the date of death or last follow-up. RFS was defined as the time duration from the date of surgery to tumor recurrence, while recurrence was defined as suspicious or confirmed lesions based on imaging or histological examination. Patients were screened for HCC recurrence by ultrasonography, contrast-enhanced CT scans and blood tests (tumor markers) every 3–4 months after surgery. After two years, the frequency of these checks was reduced to no more than once every six months. After recurrence, patients received appropriate therapeutic modalities and the same surveillance continued.

Flow cytometry

2 mL of venous blood was collected into heparin coated tubes from fasting subjects in the morning prior to hepatectomy. Two test tubes were added with 100μL of whole anticoagulant blood, one tube was added with 10 μL each of PC5-CD4, PE-CD127 and FITC-CD25, and the other tube was added with 10 μL each of PC5-CD4, FITC-IgG1 and the corresponding homologous control -PE of each monoclonal antibody. The solutions were vortexed thoroughly and then incubated in the dark at room temperature for 15 min. Subsequently, 1 mL of the hemolytic agent was added to the mixture. After mixing it well, the resulting solution was placed in a 37℃ water bath for 10 min, during which it was vortexed periodically. After carefully discarding the supernatant, the remaining cells were resuspended in phosphate-buffered saline (PBS) and then analyzed by flow cytometry.

Prior to detection, a single - staining control assay was performed to validate antibody specificity.

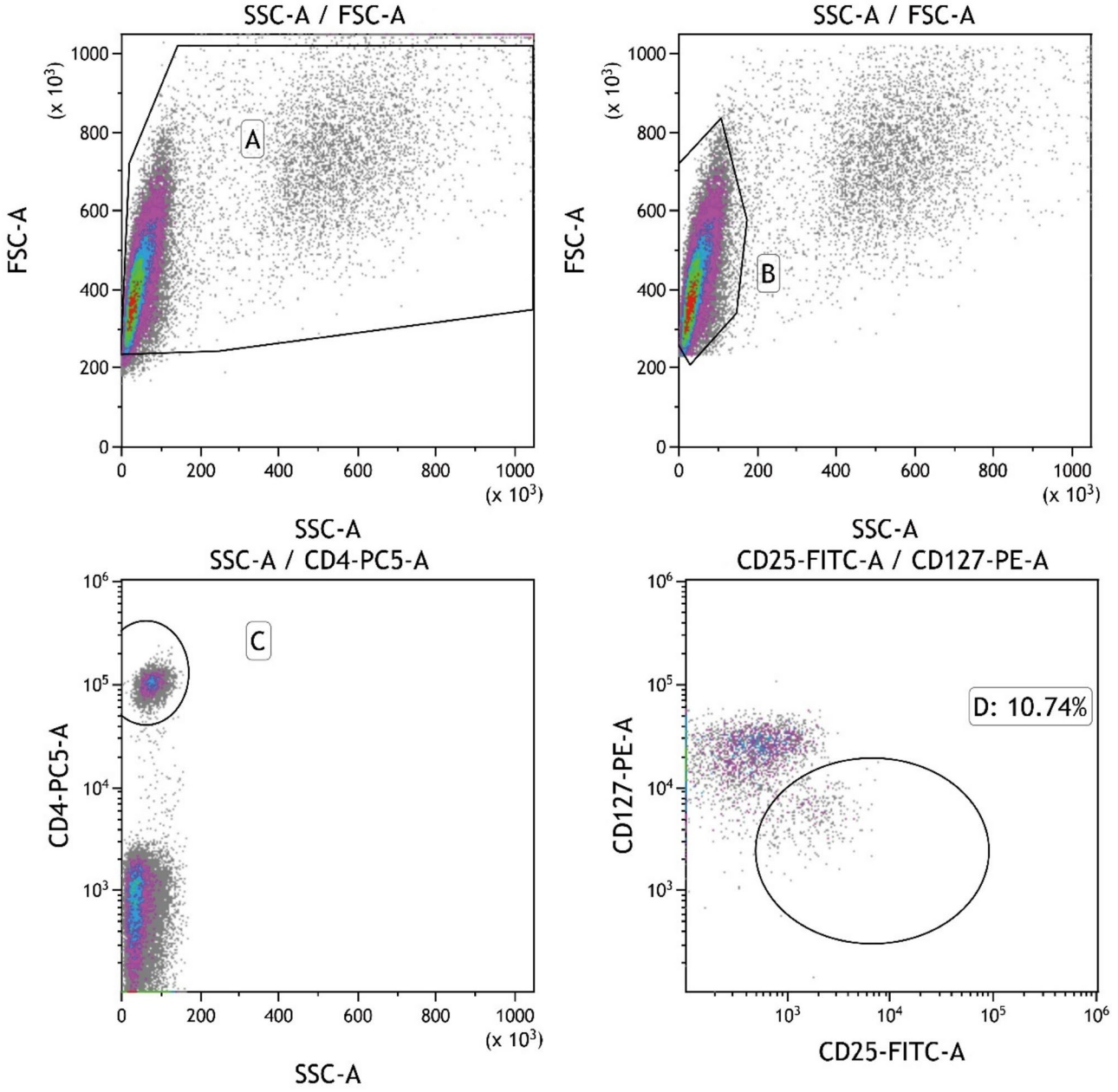

Fig. 1 Experimental analysis of CD4⁺CD25⁺CD127^{low} regulatory T cells. (**A**) Gate A was set to eliminate incompletely lysed debris. (**B**) Gate B was established to isolate lymphocytes. (**C**) Gate C was set to analyze CD4⁺ T lymphocytes. (**D**) Gate D was set to analyze the percentage of CD4⁺CD25⁺CD127^{low} regulatory T cells

Routine quality control of the optical flow path and fluorescence compensation of the flow cytometer were carried out to ensure that all the instrument's parameters were within the allowable range of quality control. During detection, lymphocyte populations were gated first according to the forward scatter (FSC) and side scatter (SSC) light signals (detailed steps in Fig. 1). For each specimen, more than 10,000 cells within the gated area were acquired. The CXP 2.0 software was used for result analysis. Cell subsets exhibiting the immunophenotype $CD4^+CD25^+CD127low$ are classified as Treg cells. $CD127^{low}$ is defined based on a percentage threshold. The percentage of $CD4^+CD25^+CD127low$ cells relative to total CD4 + T lymphocytes reflects Treg percentage. All detections and analyses were completed by the same team. All reagents and instruments were procured from Beckman Coulter, Inc. (USA).

Statistical analyses

Clinicopathological characteristics were summarized using frequencies/percentages for categorical variables, while median and interquartile range (IQR) were reported for continuous covariates. Student's t test was used for comparisons of continuous variables when applicable; otherwise, the Mann-Whitney U test was applied. Categorical variables were compared with the $\chi 2$ test or the Fisher's exact test, as appropriate. The prognostic value of $CD4^+CD25^+CD127^{low}$ Tregs were evaluated by Cox proportional hazards models and log-rank tests. The Kaplan–Meier survival curves were used to visualize the survival differences. The optimal cut-off value of Treg percentage was calculated using

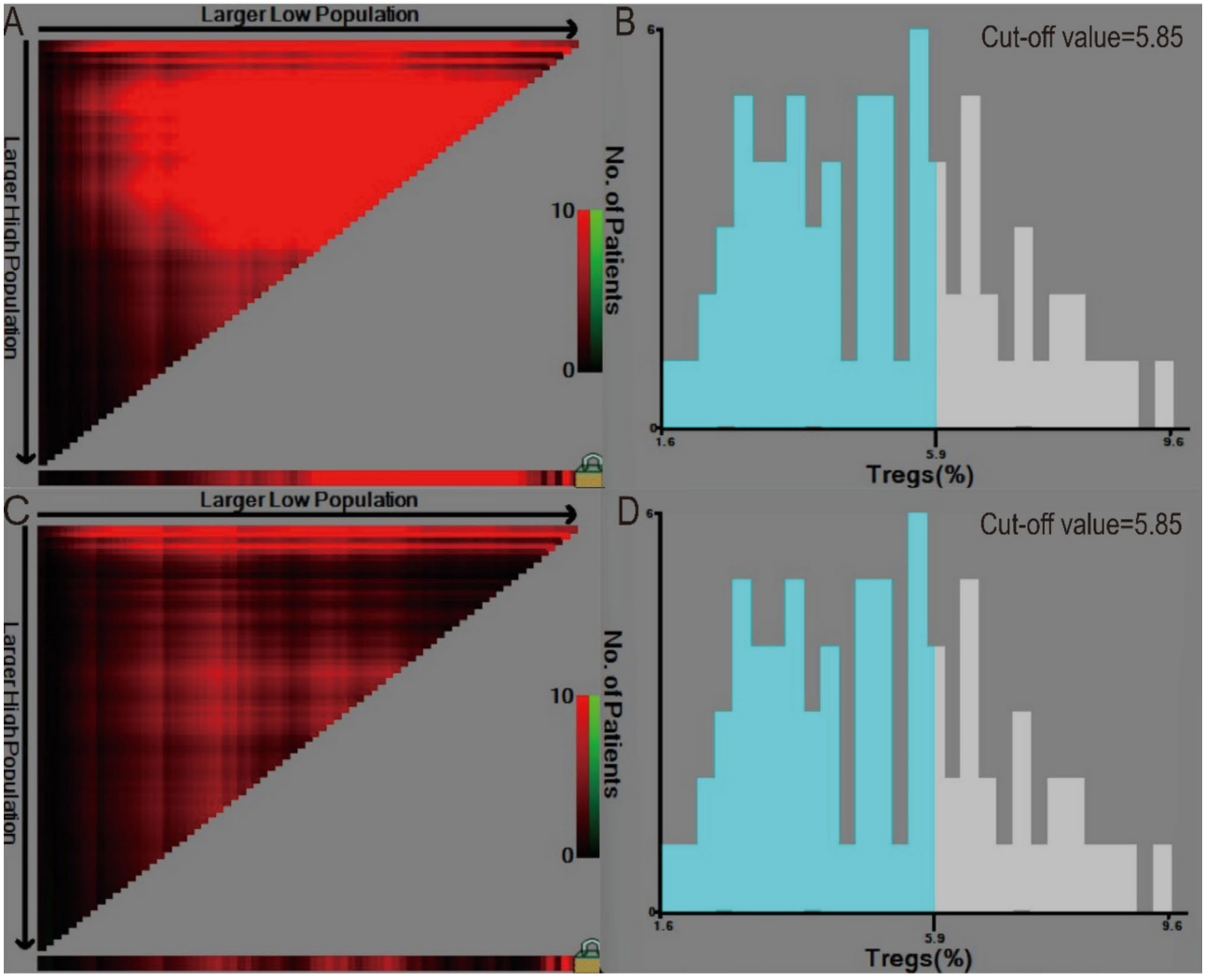

Fig. 2 The optimal cut-off value of Treg percentage in peripheral blood was determined by X-tile analysis. In terms of both RFS (**A**, **B**) and OS (**C**, **D**), the optimal cut-off value was 5.85%

Abbreviations: Treg, regulatory T cells; RFS, recurrence-free survival; OS, overall survival

Table 1 Clinicopathological characteristics of HCC patients

	Low (*N* = 51)	High (*N* = 26)	*p*-value
Sex			
female	10 (19.6%)	4 (15.4%)	0.887
male	41 (80.4%)	22 (84.6%)	
Age (Years)			
Mean (SD)	58.4 (12.4)	56.8 (10.6)	0.581
HBV.DNA			
< 1000 IU/mL	32 (62.7%)	17 (65.4%)	0.820
> 1000 IU/mL	19 (37.3%)	9 (34.6%)	
AFP			
< 400 ng/mL	31 (60.8%)	16 (61.5%)	0.949
> 400 ng/mL	20 (39.2%)	10 (38.5%)	
TBIL (μmol/L)			
Median [Min, Max]	14.2 [6.50, 141]	17.0 [8.50, 47.5]	0.200
ALB (g/L)			
Mean (SD)	39.1 (4.69)	38.7 (4.28)	0.733
PT (s)			
Median [Min, Max]	11.8 [1.10, 24.9]	12.4 [10.8, 16.1]	0.054
Operation. time (minutes)			
Median [Min, Max]	212 [93.0, 582]	224 [89.0, 392]	0.742
Blood. loss			
≤ 300 mL	40 (78.4%)	20 (76.9%)	0.880
> 300 mL	11 (21.6%)	6 (23.1%)	
Hepatectomy			
≤ 2 segmentectomy	40 (78.4%)	23 (88.5%)	0.443
> 2 segmentectomy	11 (21.6%)	3 (11.5%)	
Tumor. diameter			
< 5 cm	25 (49.0%)	12 (46.2%)	0.812
> 5 cm	26 (51.0%)	14 (53.8%)	
Satellite. nodules			
No	37 (72.5%)	22 (84.6%)	0.237
Yes	14 (27.5%)	4 (15.4%)	
MVI			
No	25 (49.0%)	15 (57.7%)	0.471
Yes	26 (51.0%)	11 (42.3%)	
Differentiation			
Medium-high	25 (49.0%)	17 (65.4%)	0.173
Low	26 (51.0%)	9 (34.6%)	

Abbreviations: HCC, Hepatocellular carcinoma; AFP, alpha-fetoprotein; PT, prothrombin time; ALB, albumin; TBIL, total bilirubin; MVI, microvascular invasion

X-Tile software (Yale University) [16], and all the other statistical analyses were conducted using R version 4.4.1. A two-tailed *p*-value less than 0.05 was considered statistically significant.

Results

Clinicopathological characteristics

In this analysis, 77 patients who had undergone hepatectomy for HCC were included. X-tile software was used to calculate optimal cut-off value of Treg percentage (5.85%), and patients were divided into two groups with high and low Treg percentage (Fig. 2). The median age of these patients was 57 years, with a predominance of males ($n = 63$, 81.8%). At initial diagnosis, the median percentage of $CD4^+CD25^+CD127^{low}$ cells in peripheral blood relative to CD4 + T lymphocytes was 4.92 (range: 1.63–9.56). Approximately one-third of the patients exhibited high levels of HBV-DNA replication, and nearly half had AFP > 400 ng/mL. Both the median and mean values of relevant liver function markers were generally within the normal range. More than two-thirds of the patients underwent hepatectomy, with two or fewer liver segments, the average operation time for patients was 231 min, and most patients suffered less than or equal to 300 mL of blood loss. Tumors larger than 5 cm in diameter were observed in more than half of the patients, and the majority of tumors were moderately to well

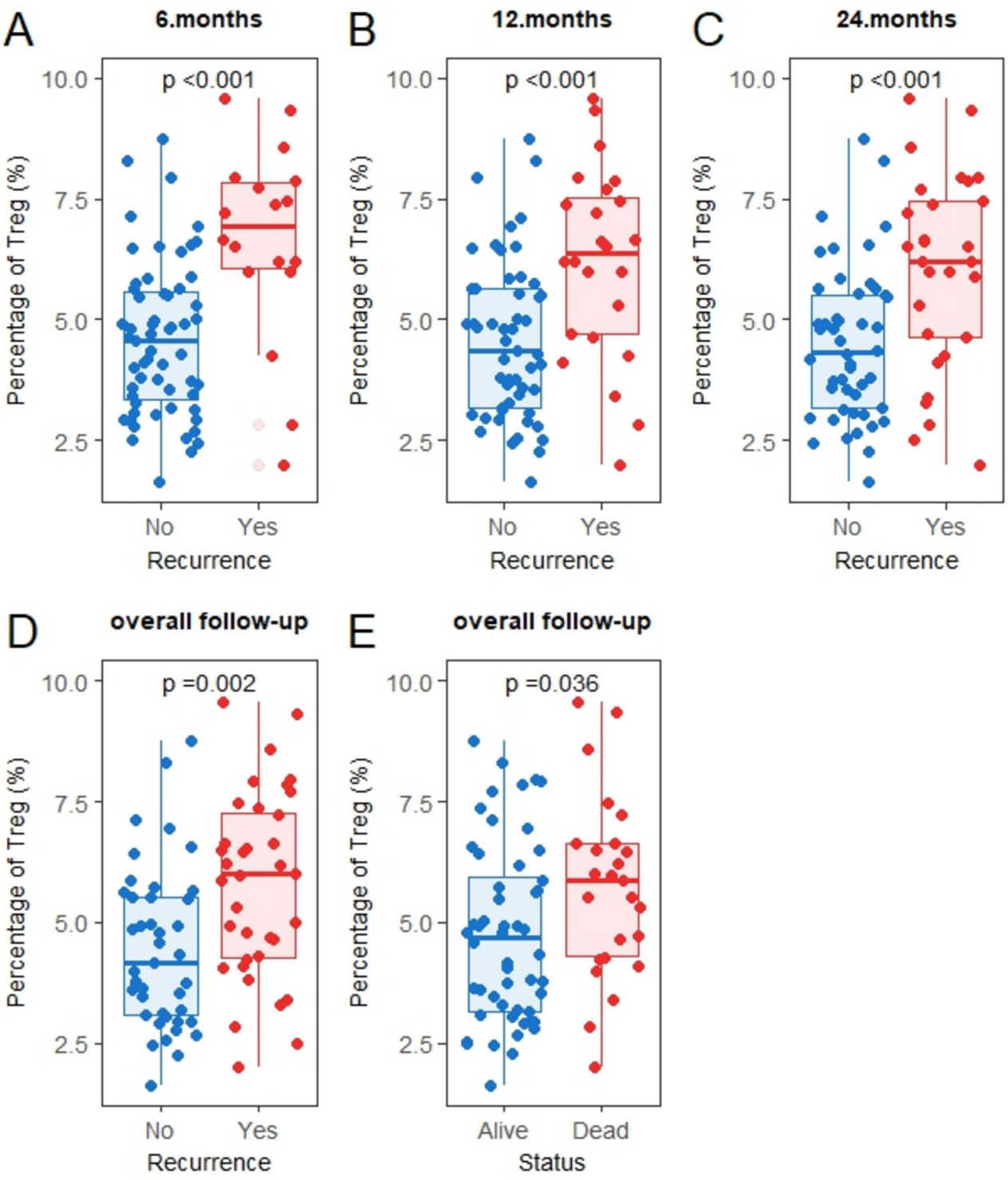

Fig. 3 The boxplot with scatter points is used to illustrate the percentage of $CD4^+CD25^+CD127^{low}$**Tregs across different subgroups of HCC patients.** (**A**): Recurrence within 6 months. (**B**): Recurrence within 12 months. (**C**): Recurrence within 24 months. (**D**): Recurrence within overall follow-up time. (**E**): Status within overall follow-up time

Abbreviations: Treg, regulatory T cells; HCC, Hepatocellular carcinoma

differentiated, with less than half showing microvascular invasion. Upon conducting a comparison of the clinicopathological data incorporated within the high Tregs group and the low Tregs group, nearly all of

Table 2 The differences in recurrence or survival rate between the low and high Treg percentage groups

	Low (*N* = 51)	High (*N* = 26)	*p*-value
Recurrence.within.6.months			
No	48 (94.1%)	11 (42.3%)	< 0.001
Yes	3 (5.9%)	15 (57.7%)	
Recurrence.within.12.months			
No	43 (84.3%)	10 (38.5%)	< 0.001
Yes	8 (15.7%)	16 (61.5%)	
Recurrence.within.24.months			
No	41 (80.4%)	7 (26.9%)	< 0.001
Yes	10 (19.6%)	19 (73.1%)	
Recurrence			
No	35 (68.6%)	6 (23.1%)	< 0.001
Yes	16 (31.4%)	20 (76.9%)	
Status			
Alive at last follow-up	39 (76.5%)	13 (50.0%)	0.019
Died	12 (23.5%)	13 (50.0%)	

Abbreviations: Treg, regulatory T cells

the *P* - values failed to reach statistical significance. Additionally, PT manifested a borderline difference ($p = 0.054$), with the high Tregs group demonstrating a marginally longer PT. Table 1 provides a detailed summary of these characteristics.

Treg percentage in peripheral blood was significantly elevated in patients who suffered poor clinical outcome

The median follow-up time after the initial curative hepatectomy was 42 months, ranging from 4 to 66 months. During the overall follow-up, 36 of 77 patients recurred, of which 29 patients (37.7%) had early recurrence, 7 patients (9.1%) had late recurrence, and 25 patients (32.5%) died. The cumulative RFS at 6 months, 12 months, and 24 months were 76.6%, 68.8%, and 62.3%, respectively. Treg percentage in peripheral blood was significantly higher in recurrent and dead patients than in non-recurrent and alive patients, recurrence within 6 months (6.93% vs. 4.57%, $p < 0.001$, Fig. 3A), recurrence within 12 months (6.36% vs. 4.35%, $p < 0.001$, Fig. 3B), recurrence within 24 months (6.21% vs. 4.32%, $p < 0.001$, Fig. 3C), recurrence within overall follow-up (5.99% vs. 4.17%, $p = 0.002$, Fig. 3D), status at last follow-up (5.87% vs. 4.69%, $p = 0.036$, Fig. 3E). Next, the differences in the recurrence and survival rates between the low and high Tregs groups were statistically significant ($p < 0.05$), with the high Tregs group exhibiting significantly higher rates than the low Tregs group (Table 2; Fig. 4).

The value of Treg percentage in prognosis assessment

The recurrent pattern was defined as early recurrence (recurrence within 24 months after the initial hepatectomy). First, univariate Cox regression was conducted. Subsequently, variables with a $p < 0.05$ were included in the multivariate Cox regression. Through univariate and multivariate analyses identified that high Treg percentage was an independent risk predictor for both RFS (HR:19.14, 95%CI: 6.63–55.21, $p < 0.001$, Table 3) and OS (HR 4.59,95%CI 1.82–11.59, $p = 0.001$, Table 4), The forest map was drawn according to the results of multivariate COX analysis (Fig. 5). We found that patients with high Treg percentage had significantly shorter RFS (the log-rank test, $p < 0.001$, Fig. 6A) and OS (the log-rank test, $p < 0.001$, Fig. 6B) than patients with low Treg percentage. We evaluated the variance inflation factors (VIFs) of all covariates. The VIF values of all variables were below the threshold of 2 (the maximum VIF was 1.839). In addition, we investigated the prognostic significance of Treg percentage in subgroups with a low risk of recurrence/death. In patients with AFP < 400 ng/mL, patients with high Treg percentage had lower RFS (the log-rank test, $p < 0.001$, Fig. 7A) and OS (the log-rank test, $p < 0.001$, Fig. 7A) than those with low Treg percentage. Among patients without MVI, high Treg percentage also indicated a significantly shorter RFS (the log-rank test, $p < 0.001$, Fig. 7B) and OS (the log-rank test, $p = 0.018$, Fig. 7B). Moreover, Treg percentage retained its prognostic value in patients with medium-high differentiation (the log-rank test, RFS: $p < 0.001$, OS: $p = 0.005$, Fig. 7C). Next, we analyzed the antitumor therapy adopted by patients with early recurrence. Four types of treatment were adopted for tumor recurrence: re-resection (4, 13.8%), TACE (15, 51.7%), targeted therapy (15, 51.7%) and immunotherapy (16, 55.2%). We compared the prognosis of these patients by univariate analysis, and the results are shown in Table 5. Notably, different therapies may have an impact on prognosis following early recurrence. Collectively, our data

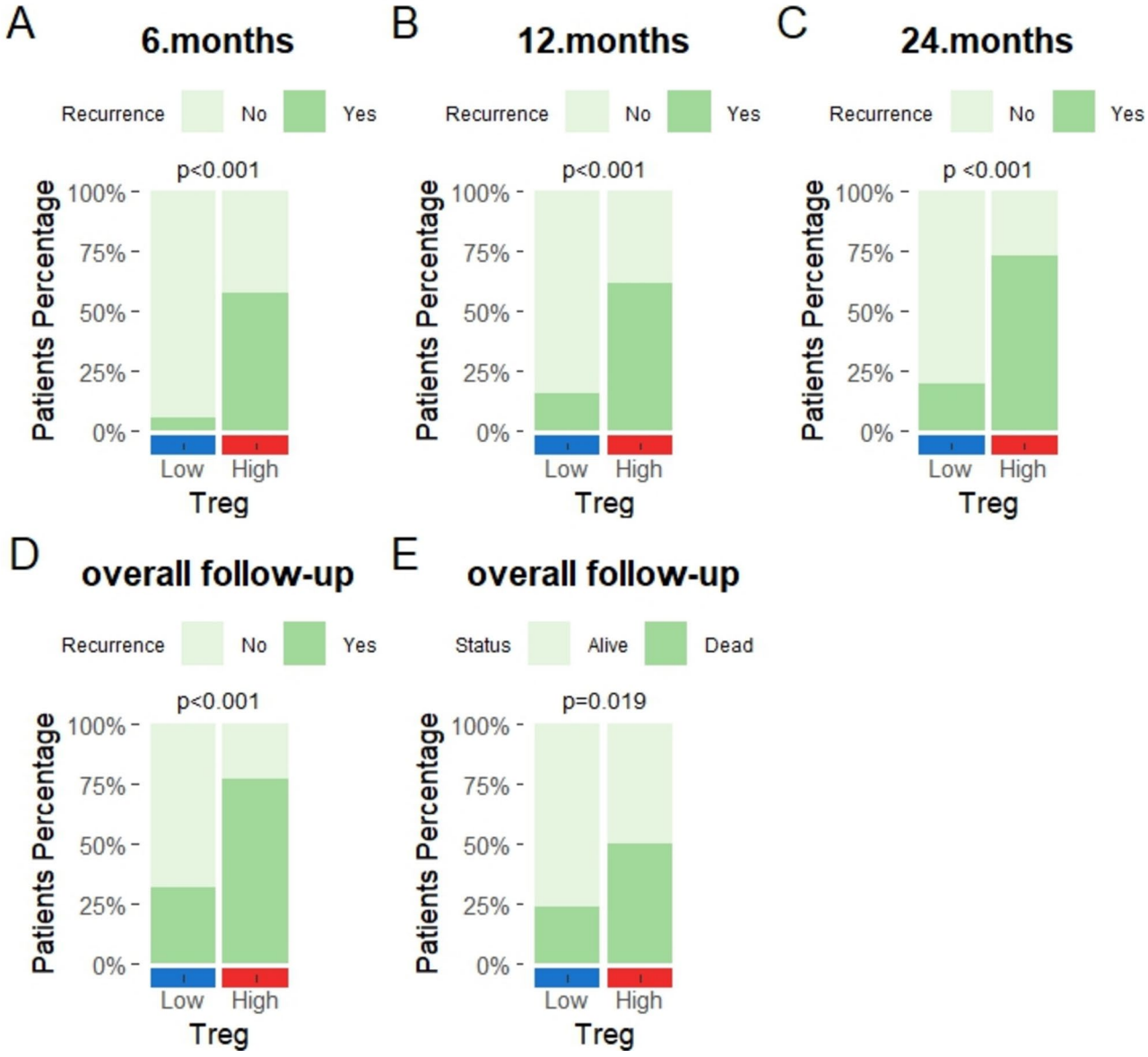

Fig. 4 Postoperative differences in recurrence and mortality between the groups with low Treg percentage and the groups with high Treg percentage. (**A**): Recurrence within 6 months. (**B**): Recurrence within 12 months. (**C**): Recurrence within 24 months. (**D**): Recurrence within overall follow-up time. (**E**): Status within overall follow-up time

Abbreviations: Treg, regulatory T cells; HCC, Hepatocellular carcinoma

effectively confirmed the prognostic value of the Treg percentage in peripheral blood for HCC.

Discussion

The survival of liver cancer patients is affected by multiple factors, and recurrence after hepatectomy is a major challenge, with an incidence as high as 50–70% [17, 18]. Thus, a large amount of research has focused on this issue, proposing various indicators for assessing recurrence risk [19, 20]. Among them, the recurrence of HCC, especially early recurrence, is closely related to tumor cells evading immune surveillance, in which Tregs play a crucial role.

Tregs have a complex relationship with the prognosis of HCC patients. They can not only suppress excessive immune responses to protect normal bodily functions but also, more importantly, promote immune tolerance, enabling cancer cells to escape immune attacks [21]. In the immune escape mechanism of HCC, Tregs inhibit the killing ability of immune cells against HCC cells through various pathways, facilitating the immune escape of cancer cells [22, 23]. Studies have reported that an increase in the peripheral Treg load

Table 3 Univariate and multivariate Cox regression analyses of early recurrence risk factors in 77 HCC patients post - hepatectomy

Characteristic		all	HR (univariable)	HR (multivariable)
Treg	Low	51 (66.2%)		
	High	26 (33.8%)	6.78 (3.13–14.72, $p < 0.001$)	19.14 (6.63–55.21, $p < 0.001$)
Sex	female	14 (18.2%)		
	male	63 (81.8%)	1.44 (0.50–4.13, $p = 0.501$)	
Age	Mean ± SD	57.9 ± 11.8	0.99 (0.96–1.02, $p = 0.605$)	
HBV.DNA	< 1000 IU/mL	49 (63.6%)		
	> 1000 IU/mL	28 (36.4%)	2.24 (1.08–4.65, $p = 0.030$)	3.48 (1.35–8.99, $p = 0.010$)
AFP	< 400 ng/mL	47 (61.0%)		
	> 400 ng/mL	30 (39.0%)	1.78 (0.86–3.69, $p = 0.123$)	
TBIL	Median [Min, Max]	14.8 [6.50, 141]	1.01 (1.00-1.03, $p = 0.041$)	1.01 (0.99–1.02, $p = 0.324$)
ALB	Mean ± SD	39.0 ± 4.5	0.92 (0.85-1.00, $p = 0.040$)	0.90 (0.80-1.00, $p = 0.060$)
PT	Median [Min, Max]	11.9 [1.1, 24.9]	1.02 (0.89–1.17, $p = 0.785$)	
Operation. time	Median [Min, Max]	212 [89.0, 582]	1.00 (1.00–1.00, $p = 0.841$)	
Blood. loss	≤ 300 mL	60 (77.9%)		
	> 300 mL	17 (22.1%)	1.52 (0.67–3.43, $p = 0.314$)	
Hepatectomy	≤ 2 segmentectomy	63 (81.8%)		
	> 2 segmentectomy	14 (18.2%)	0.65 (0.23–1.86, $p = 0.420$)	
Tumor. diameter	< 5 cm	37 (48.1%)		
	> 5 cm	40 (51.9%)	2.21 (1.03–4.76, $p = 0.043$)	0.72 (0.24–2.13, $p = 0.551$)
Satellite. nodules	No	59 (76.6%)		
	Yes	18 (23.4%)	2.65 (1.26–5.57, $p = 0.010$)	3.60 (1.23–10.56, $p = 0.019$)
MVI	No	40 (51.9%)		
	Yes	37 (48.1%)	2.89 (1.31–6.36, $p = 0.008$)	2.63 (0.98–7.02, $p = 0.054$)
Differentiation	Medium-high	42 (54.5%)		
	Low	35 (45.5%)	1.87 (0.89–3.92, $p = 0.097$)	

$n = 77$, events = 29, Likelihood ratio test = 56.07 on 7 df($p < 0.001$)

Abbreviations: HCC, Hepatocellular carcinoma; HR, hazard ratio; RFS, recurrence-free survival; Tregs, regulatory T cells; AFP, alpha-fetoprotein; PT, prothrombin time; ALB, albumin; TBIL, total bilirubin; MVI, microvascular invasion;

will replenish the intratumoral Tregs, thereby increasing the ratio of $CD4^+$ Tregs to $CD8^+$ T cells within the tumor [24]. And with the deepening of research on the peripheral immune landscape, its importance in tumor recognition and control has become increasingly prominent [25]. Given the significant impact of Tregs on immune escape, which in turn affects the recurrence and prognosis of HCC patients, this study combines peripheral blood Tregs and clinicopathological characteristics of HCC patients for prognostic analysis, aiming to better understand the role of Tregs in HCC prognosis and explore new therapeutic targets or prognostic markers.

$CD4^+CD25^+$ Tregs can be detected in peripheral blood, local tumors, invasive lymph nodes, and drainage lymph nodes of patients with various cancers, including gastric, lung, ovarian, liver, pancreatic and breast cancers. The $CD4^+CD25^+$ Tregs level in peripheral blood is negatively correlated with the disease course and prognosis; higher Treg percentage indicates a worse prognosis [26, 27], which is consistent with our study's findings. We discovered that $CD4^+CD25^+CD127^{low}$ Tregs were significantly elevated in peripheral blood of HCC patients with dismal clinical outcomes. However, predicting which individuals will exhibit a poor outcome after hepatectomy for early-stage HCC remains challenging in clinical practice [28]. Elevated Treg percentage has been reported to be associated with better survival in colorectal, head - and - neck, and esophageal cancers [29, 30]. Additionally, Tregs from different locations can lead to different prognoses. Overall, the significance of Tregs in the assessment of recurrence and prognosis in HCC patients remains controversial and needs to be further explored based on robust clinical data. Therefore, we utilized the X - tile software to calculate that the optimal cut-off value for Treg percentage was 5.85%. This is similar to previous studies regarding risk stratification and diagnosis of diseases associated with the relationship between peripheral blood Treg percentage and other tumors. For instance, in chronic lymphocytic leukemia, the cut -off value of the percentage of peripheral blood Treg percentage was 5.7% [31].

In addition, the postoperative recurrence of HCC involves various molecular mechanisms, which can be divided into early recurrence and late recurrence according to the time to recurrence (TTR). In most studies, recurrence within 2 years after surgery is

Table 4 Univariate and multivariate Cox regression analyses of overall survival risk factors in 77 HCC patients post - hepatectomy

Characteristic		all	HR (univariable)	HR (multivariable)
Treg	Low	51 (66.2%)		
	High	26 (33.8%)	2.78 (1.27–6.11, $p=0.011$)	4.59 (1.82–11.59, $p=0.001$)
Sex	female	14 (18.2%)		
	male	63 (81.8%)	2.86 (0.67–12.13, $p=0.154$)	
Age	Mean ± SD	57.9 ± 11.8	0.98 (0.95–1.02, $p=0.310$)	
HBV.DNA	< 1000 IU/mL	49 (63.6%)		
	> 1000 IU/mL	28 (36.4%)	3.28 (1.47–7.32, $p=0.004$)	3.89 (1.36–11.11, $p=0.011$)
AFP	< 400 ng/mL	47 (61.0%)		
	> 400 ng/mL	30 (39.0%)	1.54 (0.70–3.38, $p=0.279$)	
TBIL	Median [Min, Max]	14.8 [6.50, 141]	1.01 (1.00-1.03, $p=0.096$)	
ALB	Mean ± SD	39.0 ± 4.5	0.87 (0.79–0.96, $p=0.006$)	0.89 (0.79-1.00, $p=0.058$)
PT	Median [Min, Max]	11.9 [1.1, 24.9]	1.03 (0.90–1.18, $p=0.694$)	
Operation. time	Median [Min, Max]	212 [89.0, 582]	1.00 (1.00-1.01, $p=0.549$)	
Blood. loss	≤ 300mL	60 (77.9%)		
	¬ 300mL	17 (22.1%)	1.37 (0.57–3.28, $p=0.484$)	
Hepatectomy	≤ 2 segmentectomy	63 (81.8%)		
	> 2 segmentectomy	14 (18.2%)	0.47 (0.14–1.59, $p=0.224$)	
Tumor. diameter	< 5 cm	37 (48.1%)		
	> 5 cm	40 (51.9%)	2.99 (1.24–7.16, $p=0.014$)	0.98 (0.31–3.15, $p=0.973$)
Satellite. nodules	No	59 (76.6%)		
	Yes	18 (23.4%)	2.78 (1.24–6.26, $p=0.013$)	2.45 (0.84–7.14, $p=0.099$)
MVI	No	40 (51.9%)		
	Yes	37 (48.1%)	3.00 (1.28–7.01, $p=0.011$)	1.86 (0.69–4.99, $p=0.219$)
Differentiation	Medium-high	42 (54.5%)		
	Low	35 (45.5%)	2.02 (0.91–4.49, $p=0.086$)	

$n=77$, events = 25, Likelihood ratio test = 31.67 on 6 df($p<0.001$)

Abbreviations: HCC, Hepatocellular carcinoma; HR, hazard ratio; OS, overall survival; Treg, regulatory T cells; AFP, alpha-fetoprotein; PT, prothrombin time; ALB, albumin; TBIL, total bilirubin; MVI, microvascular invasion;

defined as early recurrence, while recurrence after 2 years is defined as late recurrence. Generally, the longer the TTR, the better the prognosis [32, 33]. Therefore, identifying the risk factors for early recurrence is crucial for the prevention and treatment of early HCC recurrence. In this study, through the multivariate COX regression model, we found that the percentage of $CD4^+CD25^+CD127low$ Tregs served as an independent predictor for both early recurrence and mortality in HCC patients following hepatectomy. This observation suggests that elevated peripheral blood Treg levels in HCC patients may be associated with more aggressive tumor behavior and/or a compromised immune microenvironment, potentially contributing to unfavorable clinical outcomes. However, the precise molecular mechanisms underlying the relationship between peripheral blood Tregs and clinical outcomes remain to be fully elucidated. The stability of the model remains uncertain, and this is an area worthy of our further exploration. Moreover, due to the relatively small overall sample size, we obtained wide confidence intervals. The stability of the model remains uncertain, and this is an area worthy of our further exploration.

Subsequently, we conducted a more detailed analysis of the prognostic value of Tregs. We used the percentage of Tregs in subgroups defined by AFP, MVI, and tumor histological differentiation grade to evaluate the prognosis of HCC patients in the low - recurrence/mortality - risk subgroups. The results of these subgroup analyses further validated the prognostic value of the percentage of peripheral blood Tregs. In recent years, advancements in interventional, targeted and immunotherapy have improved the survival rates of HCC patients after hepatectomy [3, 34]. We further asked whether the prognosis of early-recurrent patients might differ by Treg percentage depending on the type of post-recurrence therapy. To investigate whether different treatments impact the prognosis of HCC patients after early recurrence, we analyzed the therapies administered to patients with early recurrence. We found that different therapies may have an impact on prognosis after early recurrence. However, considering the small number of patients with recurrence included in this study, we did not further explore the correlation between different postoperative treatment results and Treg percentage, which is also the direction of further research of our team.

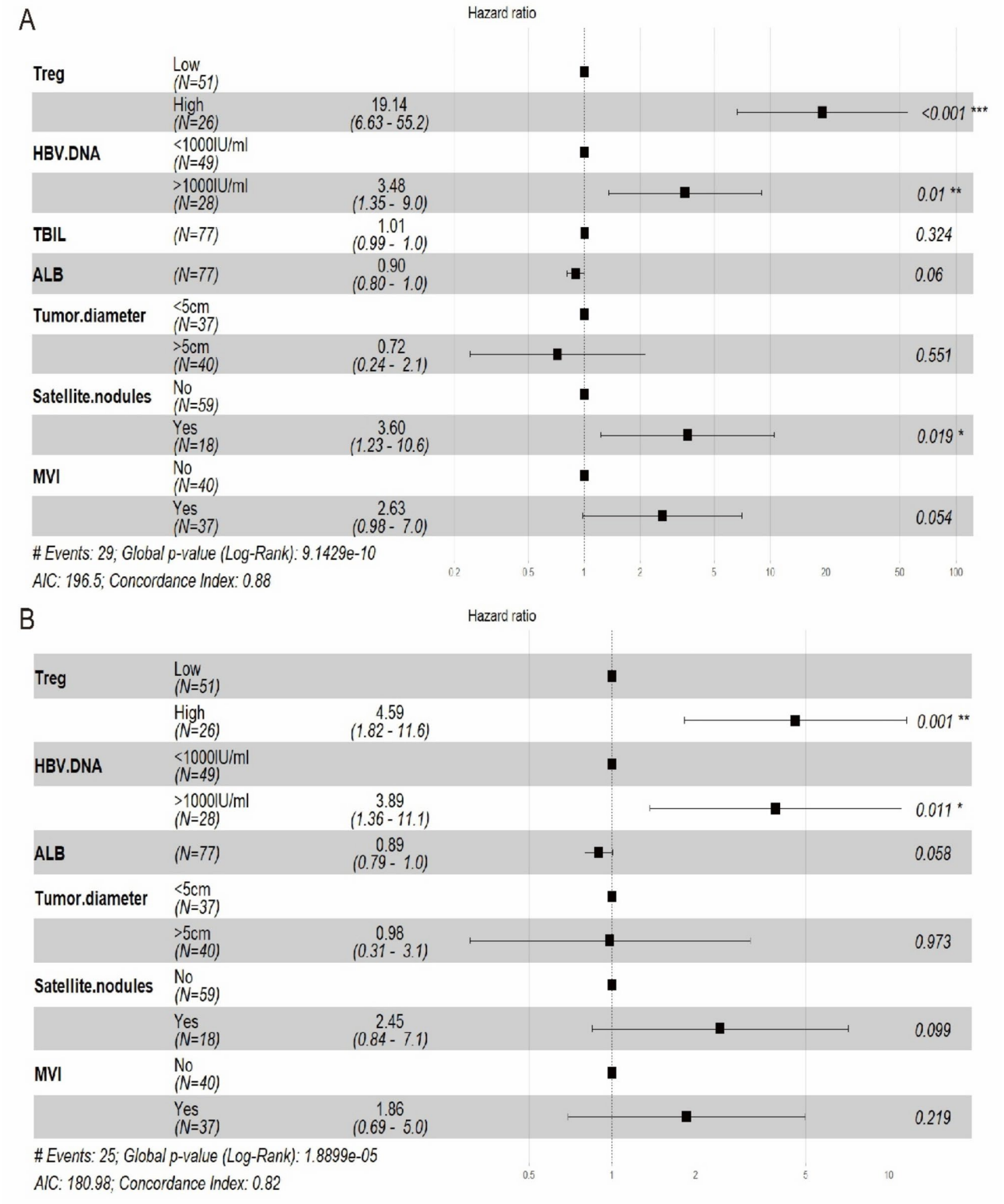

Fig. 5 Forest plot illustrating the results of multivariate Cox regression analyses for early recurrence (**A**) and overall survival (**B**)
Abbreviations: Treg, regulatory T cells; ALB, albumin; TBIL, total bilirubin; MVI, microvascular invasion; AIC, Akaike information criterion

In this study, the cumulative RFS at 6, 12, 24 months and overall follow-up time were 76.6%, 68.8%, 62.3% and 53.2%, respectively. The overall OS rate during the entire follow-up was 67.5%, which is similar to what is

reported by many other series [35, 36]. All the clinicopathologic characteristics in this study have been thoroughly validated by clinical and basic medical research. They are closely associated with liver physiology and pathology. Notably, the VIFs of all covariates are well below 2. For instance, liver - function - related markers such as ALB and TBIL likely play a crucial role in tumor growth, metastasis, and treatment responses, and thus are included as potential factors in model construction [1, 2, 3]. This study identified HBV-DNA > 1000 IU/mL as an independent risk factor for both early recurrence and death after hepatectomy, echoing the findings of several other studies [37, 38, 39]. Hepatitis B virus (HBV) is the most significant factor associated with the development of HCC [40], Studies on antiviral therapy [41, 42] have demonstrated the association between nucleoside or nucleotide analogues and delayed disease progression, as well as a reduced incidence of HCC. Furthermore, among patients with developed HCC, concomitant antiviral treatment after initial tumor ablation is associated with improved survival and a reduced rate of tumor recurrence [43]. Therefore, antiviral treatment is important in the comprehensive management of HBV–related HCC.

However, it is important to acknowledge several limitations in the present study. Given the single - institution nature and small sample size, multi - center studies with larger samples are needed to confirm these findings. Notably, the majority of our enrolled patients had an HBV background, which means that the clinical significance needs to be further validated in patients with other etiology backgrounds [44]. Furthermore, due to the small number of recurrent patients in this study, we did not further explore the correlation between post - recurrence treatment efficacies and Treg percentage. As only pre - operative Treg percentage was included, future research may track Treg percentage dynamics over time or after antiviral/immunotherapy interventions. These are the areas our research team is further exploring.

Conclusion

This study has for the first time demonstrated that the proportion of peripheral blood $CD4^{+}CD25^{+}CD127^{low}$ Tregs serves as a potential prognostic predictor for HCC patients following hepatectomy. It can effectively

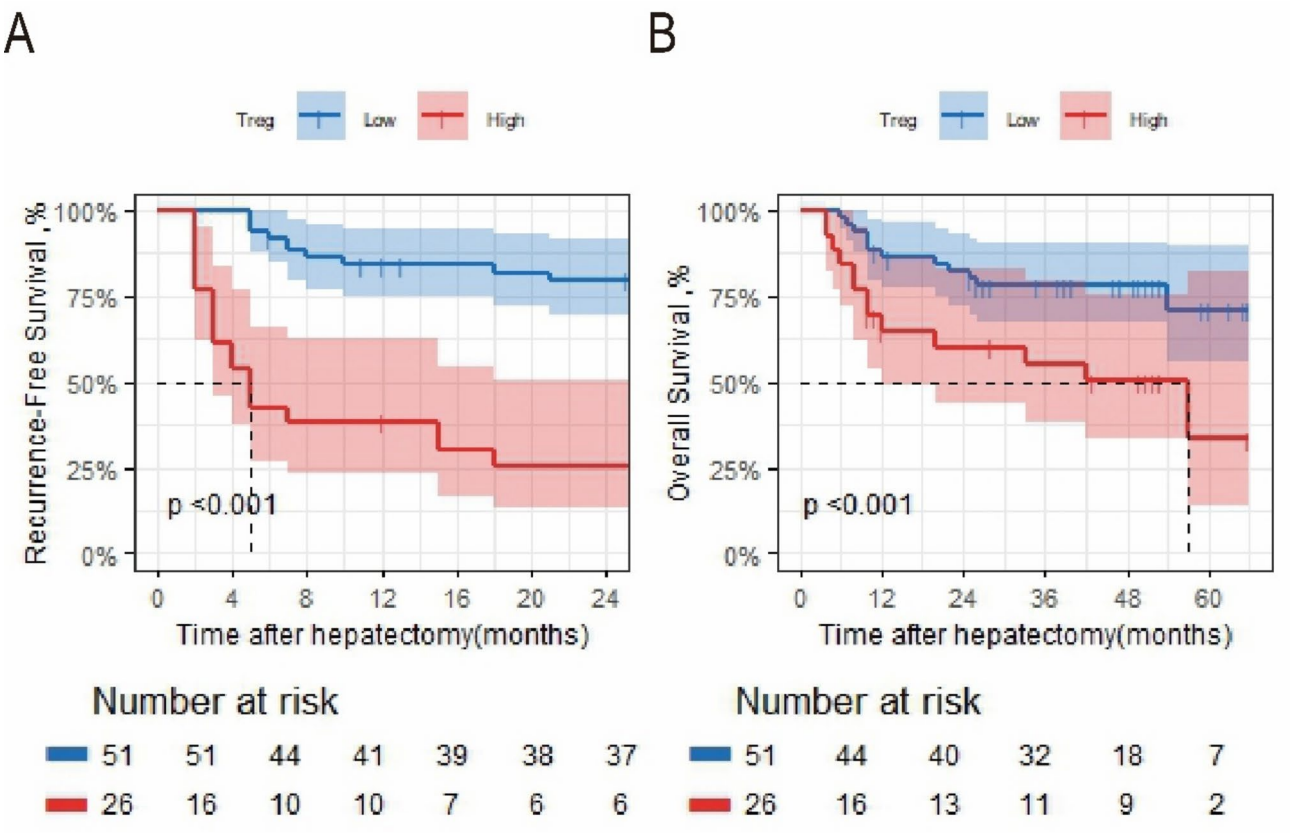

Fig. 6 Kaplan - Meier plots for comparing RFS (**A**) and OS (**B**) of HCC patients between the low and high Treg percentage groups
Abbreviations: Treg, regulatory T cells; RFS, recurrence-free survival; OS, overall survival; HCC, Hepatocellular carcinoma

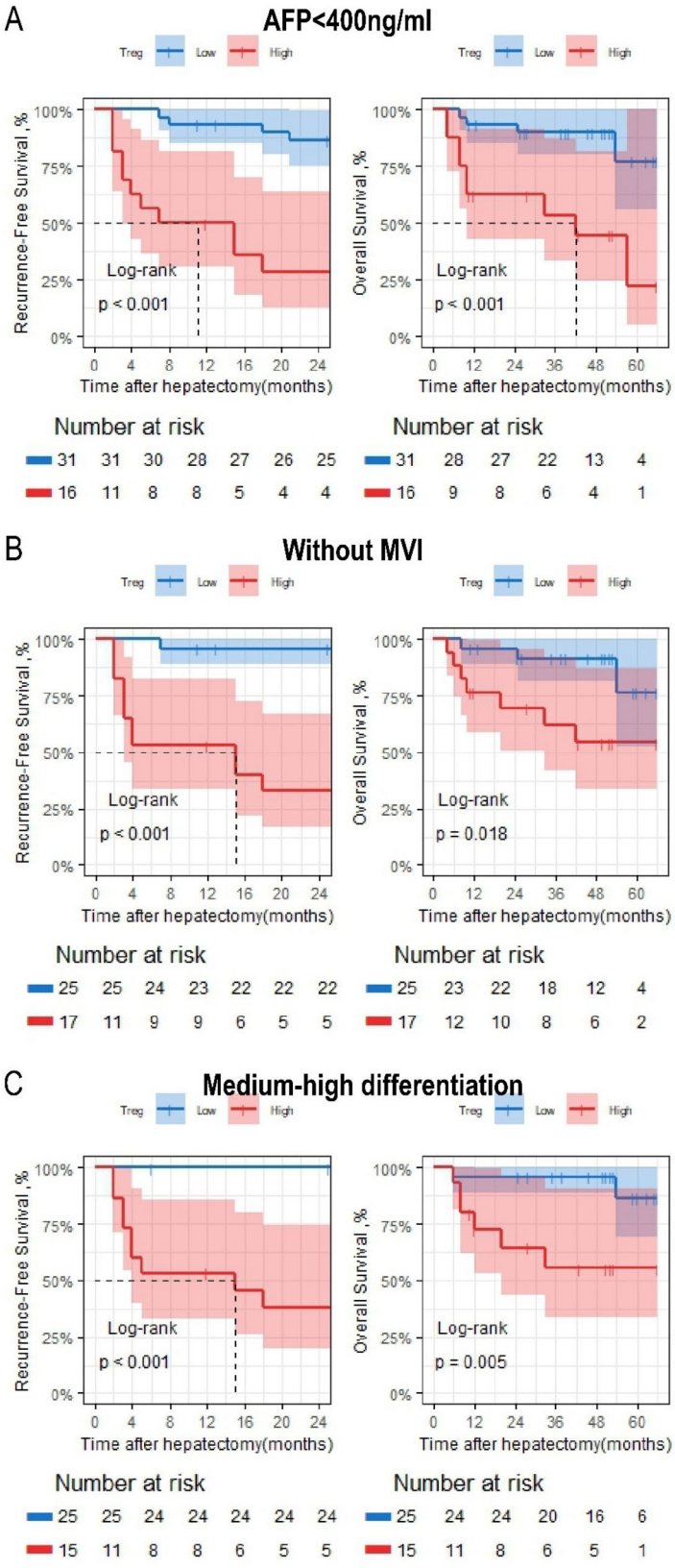

Fig. 7 Subgroup analysis: Kaplan-Meier plots comparing RFS and OS of HCC patients with AFP < 400 ng/mL (**A**), without MVI (**B**), and with medium-high differentiation (**C**) between the low and high Treg percentage groups

Abbreviations: RFS, recurrence-free survival; OS, overall survival; Treg, regulatory T cells; HCC, Hepatocellular carcinoma; AFP, alpha-fetoprotein; MVI, microvascular invasion

Table 5 Postoperative different treatments of early recurrent HCC patients according to different status

	Alive (*N* = 9)	Dead (*N* = 20)	*p*-value
TACE			
No	1 (11.1%)	12 (60.0%)	0.020
Yes	8 (88.9%)	8 (40.0%)	
Targeted.therapy			
No	5 (55.6%)	8 (40.0%)	0.688
Yes	4 (44.4%)	12 (60.0%)	
Immunotherapy			
No	4 (44.4%)	8 (40.0%)	1.000
Yes	5 (55.6%)	12 (60.0%)	
Re.hepatectomy			
No	6 (66.7%)	19 (95.0%)	0.076
Yes	3 (33.3%)	1 (5.0%)	

Abbreviations: HCC, Hepatocellular carcinoma; TACE, transcatheter arterial chemoembolization; Re. hepatectomy, repeated hepatectomy

complement the existing risk - stratification tools for HCC patients and provide novel insights and valuable clinical implications for the management of HCC patients after hepatectomy.

Abbreviations

Treg	Regulatory T cell
HCC	Hepatocellular carcinoma
HR	Hazard ratio
OS	Overall survival
RFS	Recurrence-free survival
PT	Prothrombin time
ALB	Albumin
TBIL	Total bilirubin
AFP	Alpha-fetoprotein
MVI	Microvascular invasion
PBS	Phosphate-buffered saline
FSC	Forward scatter
SSC	Side scatter
AIC	Akaike information criterion
IQR	Interquartile range
TACE	Transcatheter arterial chemoembolization
TTR	Time to recurrence
HBV	Hepatitis B virus
Re. hepatectomy	repeated hepatectomy

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Author contributions
All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Haoran Sun, Zepeng Cao, Baochen Zhao, Dachen Zhou, Zhongbiao Chen, Bin Zhang. The first draft of the manuscript was written by Haoran Sun, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability
The datasets analyzed in this study are available from the corresponding authors upon reasonable request.

Declarations

Ethics approval and informed consent
The institutional review boards of The Second Affiliated Hospital of Anhui Medical University approved this retrospective study (ethical code: YX2024-201). All participants gave their informed consent to enroll in the study. This study followed STROBE guidelines for observational studies and the 1964 Declaration of Helsinki and its later amendments.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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